

**REMARKS**

Claims 2-10, 27-31 and claims 33-53 are pending and under examination in the above-identified application. Claims 1, 11-26, and 32 have been previously canceled. Claims 2, 6, 8, 31, 38-51 have been canceled by the current amendments. Applicants reserve the right to pursue any canceled claims in a later filed application claiming priority the benefit of priority to the above application. Claims 3, 5, 7, 9, 27-30, 33-35, 52 and 53 have been amended. New claims 54-60 have been added. Claims 52 and 53 have been renumbered to correct a numbering error identified by the Examiner. Following entry of the amendments claims 2-10, 27-31, 33-37, and 52-60 will be under examination.

Claims 3, 5, 7 and 9 have been amended to update dependencies to respective independent claims. Claims 27-30 and 33-35, 52 and 53 have been amended to clarify that the first and second oligonucleotides cleaved from substrate and hybridized to the first and second target nucleic acids have different sequences. Claims 27-30, 33-35, 52 and 53 have been further amended to recite the term “probe oligonucleotides” to distinguish the first and second oligonucleotides used in the contacting step for the first and second modified oligonucleotides from the first and second different oligonucleotides initially cleaved from a substrate. Claims 27-30 and 33-35 have been further amended to recite particular assays reflected by the modification steps that can be applied to the pool of first and second oligonucleotides. Claims 52 and 53 have been further amended to recite that the substrate-bound different first and different second oligonucleotides, used to capture the first and second oligonucleotides bound to the nucleic acid targets, are randomly distributed on the surface of the substrate. Finally, claim 52 has been amended to read “contacting said first and second oligonucleotides in contact with said first and second target nucleic acids.” New independent claims 54-56 correspond to claims 27-29, with the further limitation of the random distribution of the substrate-bound different first and different second oligonucleotide being distributed randomly on the substrate surface.

Support for the amendments to claims 2, 27-30, 33-35, 52-53, and new claims 54-56 for the first and second different oligonucleotides having different sequences can be found in original claims and throughout the application as filed. For instance, canceled claim 11, as originally filed, recited a population of oligonucleotides containing at least two subpopulations

further composed of at least two different oligonucleotides of known sequence. Further, the specification, on page 8, paragraph 7, continuing to the top of page 9, defines a preferred embodiment of a pool of oligonucleotides as being composed of “two or more different oligonucleotides.” The specification on page 9, in paragraph 1, defines the population of oligonucleotides as being made up of subpopulations, with the oligonucleotides within a subpopulation being either identical or different from each other.

Support for the new claims 57 and 58 for the step of modification encompassing sequencing or amplification and the recited assays can be found throughout the application as filed, for example, at page 21, first paragraph, where the application teaches, for example, genotyping, single nucleotide polymorphism (SNP) detection, and at page 23, second paragraph, where the application teaches, for example, genotyping assays, including assays such as OLA, single base extension, Invader, and the like, assays for detection of single nucleotide polymorphisms and sequencing.

Support for the amendments to claims 27-30, 33-35, 52 and 53 for the use of probe oligonucleotides bound to a substrate to capture the modified oligonucleotides, those bound probe oligonucleotides having sequences different from the released oligonucleotides used to make the modified oligonucleotides is found in the application as filed. In particular, support for the use of probe oligonucleotides can be found on page 23, second through fourth paragraphs, where the application teaches that once formed, the pool of oligonucleotides can be used in a number of assays and that “[o]nce the solution phase is performed, the experiments may include an array detection step.”

Support for the amendments to claims 52 and 53 and new claims 54-56 for the random distribution of the populations of oligonucleotides used to capture the first and second oligonucleotides can found throughout the application as filed, including, for example, at page 17, at paragraph 3, which describes substrates with randomly distributed discrete sites for binding nucleic acids or entire surfaces that can bind nucleic acids at any position on that surface.

Support for the amendment to claim 52 for contacting the first and second oligonucleotides in contact with said first and second target nucleic acids is supported in the

specification. In particular, support for the use of oligonucleotides having different sequences can be found on page 23, second through fourth paragraphs, where the application teaches that once formed, the pool of oligonucleotides in a number of assays and that “[o]nce the solution phase is performed, the experiments may include an array detection step.”

Finally, claims 52 and 53 have been renumbered in accordance with the Examiner’s comments in paragraph 1 of the Office Action mailed June 27, 2007. Accordingly, none of the amendments raise an issue of new matter and entry thereof is respectfully requested.

Applicants have reviewed the rejections set forth in the Office Action mailed June 27, 2007 and appreciate the Examiner’s reconsideration and withdrawal of the rejection over 35 U.S.C. § 102(e) and one rejection over 35 U.S.C. § 103(a). Applicants respectfully traverse all remaining grounds for rejection.

#### **Rejections Under 35 U.S.C. § 112, First Paragraph**

The Examiner has rejected claims 2-10, 27-31, and 33-53 over 35 U.S.C. § 112, first paragraph, for allegedly containing new matter on grounds that, as described in paragraph 4 of the Office Action, the substrate-bound oligonucleotides in the second contacting step could allegedly be interpreted as being either the same as or different from the oligonucleotides cleaved from the substrate. According to the Action, if interpreted as being the same oligonucleotides, the claims contain new matter. While not conceding the merit of this rejection, independent claims 27-30, 33-35, 52 and 53 have been amended to indicate that the substrate-bound oligonucleotides in the second contacting step have different sequences from the first and second oligonucleotides cleaved from the substrate. Accordingly, removal of the rejection of claims 2-10, 27-31, and 33-53 over 35 U.S.C. § 112, first paragraph, as allegedly indefinite

#### **Rejections Under 35 U.S.C. § 112, Second Paragraph**

##### *Regarding claims 2-10, 27-31 and 33-53*

The Examiner has rejected claims 2-10, 27-31 and 33-53 as indefinite in claims 27-30, 33-35, 52 and 53 under 35 U.S.C. § 112, second paragraph, because it is allegedly unclear whether the oligonucleotides in the second contacting step are the same or different from the

oligonucleotides released from the substrate. While not conceding the merit of the rejection, claims 27-30, 33-35, 52 and 53 have been amended to indicate that the substrate-bound oligonucleotides in the second contacting step have different sequences from the first and second oligonucleotides cleaved from the substrate.

*Regarding claim 52*

The Examiner has further rejected claim 52 as indefinite under 35 U.S.C. § 112, second paragraph, because it is allegedly rendered unclear over the recitation of “contacting said first and second target nucleic acid in contact with said first and second target nucleic acids.” While not conceding the merit of the rejection, claim 53 has been amended to indicate that the first and second oligonucleotides are in contact with the first and second target nucleic acids during the second contacting step.

**Rejections Under 35 U.S.C. § 103**

Claims 2, 5-10, 27-31 and 33-51 stand rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Pastinen et al., Genome Res., vol. 7, pp. 606-614, 1997, and Lipshutz et al., U. S. Patent No. 6,013,440, in light of Sinha et al., Nucl. Acids Res., vol. 2, pp.. 4539-4557. The Examiner asserts that Pastinen et al. allegedly teaches multiplex amplification of multiple targets using primer pairs for the detection of SNPs. Lipshutz et al. is alleged to teach multiplex detection of target nucleic acids using pools of oligonucleotides cleaved from a substrate, but allegedly teaches the use synthesizing the oligonucleotides on controlled pore glass, not beads. Finally, the Examiner alleges that the Sinha et al. teaches the synthesis of oligonucleotides on CPG beads. One skilled in the art, it is alleged, would know to combine these references so as to render the claimed invention obvious.

Applicants respectfully point out that there are at least two deficiencies with regard to the aforementioned rejection: (1) The Examiner does not articulate the rationale underlying the instant rejection as required by the U.S. Supreme Court's decision in *KSR Int'l Co. v. Teleflex Inc.* and articulated in the Examination Guidelines for Determining Obviousness under 35 U.S.C. 103 in View of the Supreme Court Decision in KSR International Co. v. Teleflex Inc., 72 Fed. Reg. 57,526 (2007) (“Guidelines”) and (2) the references teach away from the claimed invention.

The U.S. Patent and Trademark Office recently promulgated guidelines for Examiners in making obviousness determinations in view of the U.S. Supreme Court's decision in *KSR Int'l Co. v. Teleflex Inc.* Examination Guidelines for Determining Obviousness under 35 U.S.C. 103 in View of the Supreme Court Decision in KSR International Co. v. Teleflex Inc., 72 Fed. Reg. 57,526 (2007) ("Guidelines") One important feature of the Guidelines is an *explicit requirement* that an Examiner provide articulated reasons for the factual determinations underlying an asserted prima facie case of obviousness. This focus is consistent with the rule set down in the KSR decision that a factfinder must provide "reasons" why an invention would have been obvious to one of ordinary skill in the art. ." *KSR* at 1741. In explicating this aspect of the Supreme Court's decision, the Guidelines set forth several different rationales that can be used to support an obvious rejection. The Guidelines further set forth explicit factual findings that an Examiner must articulate to support an obviousness rejection under each rationale. In the present case the Examiner has applied the "teaching, suggestion or motivation" test, identified in the guidelines as rationale (G). For an obviousness rejection based on this rationale for combining references, the Examiner *is required to articulate* the following: (1) a finding that there was some teaching, suggestion, or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to combine reference teachings; (2) *a finding that there was reasonable expectation of success*; and (3) whatever additional findings based on the Graham factual inquiries may be necessary, in view of the facts of the case under consideration, to explain a conclusion of obviousness. The present Office Action fails to make any mention, let alone articulate, a finding that there would have been a reasonable expectation of success when combining the cited references.<sup>1</sup> As set forth in further detail below, one of ordinary skill in the art would not have had a reasonable expectation of success in arriving at the claimed methods by combining the cited references because using a mixture of oligonucleotides obtained from the methods of Lipshutz et al. as primers in the PCR method of Pastinen et al. would not have been expected to produce desirable amplification results. Rather, the complex mixture of primers produced by Lipshutz et al. would produce spurious amplification products.

The Examiner bears the burden of establishing a *prima facie* case of obviousness. *In re Rijckaert*, 9 F.3 1531, 1532, (Fed. Cir. 1993). Only if this burden is met does the burden of

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<sup>1</sup> Notably, this deficiency applies to each of the three separate obviousness rejections set forth in the Office Action.

coming forward with rebuttal argument or evidence shift to the applicant. *Id.* at 1532. When the references cited by the examiner fail to establish a *prima facie* case of obviousness, the rejection is improper and will be overturned. *In re Fine*, 837 f.2d 1071, 1074 (Fed. Cir. 1988). In this case, the Examiner has not met this burden. Accordingly, the obviousness rejection is respectfully submitted to be deficient and the Examiner, if not removing this rejection entirely based on the deficiencies described herein, should be precluded from making the instant rejection final.

“A reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the applicant.” *In re Gurley*, 27 F.3d 551, 553 (Fed. Cir. 1994); see *KSR*, 127 S. Ct. 1727 (2007), at 1739–40 (explaining that when the prior art teaches away from a combination, that combination is more likely to be nonobvious). Additionally, a reference may teach away from a use when that use would render the result inoperable. *McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1354 (Fed. Cir. 2001).

In setting forth the rejection, the Office Action alleges that it would have been obvious to use the oligonucleotide pools of Lipshutz et al. as primers in the mutation detection method of Pastinen et al. The Office Action goes on to allege that the motivation to make this modification can be found either in Lipshutz et al. or in Pastinen et al. Applicants will address either rationale in order.

The Office Action alleges that the motivation to modify the minisequencing methods of Pastinen can be found in Lipshutz et al. where the ability of the pools to bind to substantially every known nucleic acid sequence in a sample is described. However, the mere assertion that the pools have a high complexity with regard to sequence complementarity does not provide motivation to use the pools as primers in a PCR method. On the contrary, Lipshutz et al. provides no description of using the oligo pools as primers. The only description in Lipshutz et al. of using the pools in any PCR method is to use them as *templates* for PCR. Any motivation to use the pools as primers in the minisequencing methods of Pastinen et al. in an attempt to arrive at the claimed invention would have to, therefore, come from Pastinen et al. As set forth below, Pastinen et al. does not provide this motivation and, in fact, teaches away from using the pools of Lipshutz et al. as primers in the minisequencing methods.

The Office Action alleges that the motivation to modify the minisequencing methods of Pastinen can be found in the following statement from the first paragraph at page 610 of Pastinen et al.

Despite the proceeding technical development related to miniaturized arrays for genotyping, a great challenge for the assays still lies in the sample preparation. At present, amplification of DNA templates by PCR limits the number of genomic fragments that can be analyzed efficiently. Methods are required by which a significantly larger number of fragments can be amplified or in which an amplification step is avoided.

The Examiner goes on to conclude “[t]herefore, using oligonucleotide pools of Lipshutz et al. would allow efficient multiplex amplification of target nucleic acids used for subsequent detection of SNPs.” However, this conclusion does not follow from the description in Pastinen et al. nor any other art of record. All that Pastinen et al. is stating in the passage cited by the Office Action is that there are limits to the number of genomic fragments that can be produced using PCR methods. Any conclusion that the PCR challenges had anything to do with the availability of primers can not be derived from the cited passage of Pastinen et al. In fact, at the time of the Pastinen et al. publication, PCR primers were readily available from several commercial suppliers.

In contrast to the erroneous conclusion drawn in the Office Action, the challenge referred to by Pastinen et al. was the difficulty in properly amplifying large numbers of different genomic fragments using pools of PCR primers. That this was the challenge alluded to by Pastinen et al. is apparent from a review article authored by the corresponding (i.e. senior) author of the Pastinen et al. article, Ann-Christine Syvänen. Specifically, Applicant provides Syvänen *Nature Reviews* 2:930-942 (2001) as Exhibit A. The Syvänen review describes several SNP genotyping methods including the minisequencing method described in Pastinen et al. (cited as reference 37 in the section spanning pages 931-933 of Syvänen). The Syvänen review describes the challenge of using PCR in combination with genotyping methods such as mini sequencing at page 939, last paragraph:

In practice, the requirement of a PCR amplification step to achieve sensitive and specific SNP genotyping is the principal factor that limits the throughput of assays today. Multiplex PCR amplification of more than ten DNA fragments is

difficult to carry out reproducibly owing to the generation of spurious amplification products<sup>39,41,42,80</sup>.

Accordingly, it is apparent that the challenge of using PCR in the minisequencing methods of Pastinen et al. was the generation of spurious results in multiplex formats where primers were present in pools. This refutes the motivation relied upon by the Office Action. Furthermore, the teaching of Pastinen et al., as evidenced by Syvänen, establishes that one of ordinary skill in the art would not have had a reasonable expectation of success in using the oligo pools of Lipshutz et al. as PCR primers in the minisequencing methods of Pastinen et al. Absent establishing the requisite motivation to combine, the Office has not established a *prima facie* case of obviousness.

Furthermore, Pastinen et al., as evidenced by Syvänen, teaches away from the combination alleged by the Office Action and therefore teaches away from the claimed invention. As set forth above, those of ordinary skill in the art would have understood from Pastinen et al. that pools of primers could not be used in the PCR amplification methods required for minisequencing, especially pools of more than 10 primer pairs since this would result in spurious amplification products. As described by Lipshutz et al. their methods produce oligo pools that are larger and more complex than those that can be handled by the PCR methods of Pastinen et al. Specifically, Lipshutz et al. describes their methods as producing pools having at least 20 oligonucleotides having different predetermined nucleic acid sequences (see column 2, lines 19-24 and column 3, lines 20-23). According to the teaching of Pastinen et al., as evidenced by Syvänen, a pool of this complexity would not work in the minisequencing methods of Pastinen et al. Therefore, the art teaches away from the alleged combination relied upon by the Examiner to arrive at the claimed invention.

For the above-articulated reasons, it is respectfully submitted that the combination of Pastinen et al. and Lipshutz et al., as evidenced by Sinha et al. cannot render claims 2, 5-10 and 33-51 obvious and withdrawal of this ground of rejection is respectfully requested.

Claims 3 and 4 stand rejected under 35 U.S.C. § 103 as being unpatentable over Pastinen et al. and Lipshutz et al., as evidenced by Sinha et al., and further in view of Nelson et al. (Nucl. Acids Res., vol. 20, pp 6253-6259 (1992)). Claims 3 and 4 depend from claims 27-30, and the



reasons for the rejection of claims 27-30 are incorporated into this rejection. The Examiner alleges that Lipshutz et al. teaches fluorescence teaches fluorescent detection of hybrids, while Nelson et al. teaches labeling of oligonucleotides during the synthetic steps using phosphoramidites.

For the reasons articulated above, the combination of Pastinen et al. and Lipshutz et al., as evidenced by Sinha et al., cannot make claims 27-30 obvious. Thus, the further combination in light of Nelson et al., cannot render claims 3 and 4, which depend from claims 27-30, obvious and withdrawal of the rejection is respectfully requested.

Claims 52 and 53 stand rejected under 35 U.S.C. § 103 as being unpatentable over Beattie et al., U.S. Patent No. 6,268,147 ('147 patent), and Lipshutz et al., as evidenced by Sinha et al. The Examiner alleges that Beattie et al. teaches contacting multiple nucleic acid targets simultaneously with multiple detection probes and detection of hybrids through binding of the hybrids to arrays of capture probes. Lipshutz et al. allegedly teaches pools of oligonucleotides by cleaving oligonucleotides from an array.

Applicants respectfully point out that the effective filing date for the above-identified application falls after the filing date of the provisional application upon which the '147 patent relies for its effective filing date, but precedes the filing date of the utility application that matured into the '147 patent. Therefore, unless the Examiner can show that the subject matter relied on for the instant rejection is present in the provisional application, Applicants submit that this rejection is not proper. Applicants reserve the right to amend any arguments regarding the merit of this rejection pending inspection of the priority document, Provisional Application No. 60/149,344.

Without conceding the merit of the rejection above, the applicant has amended claims 52 and 53 to include a further limitation of an array of a randomly distributed population of probe oligonucleotides for contacting with the complex of the first and second oligonucleotides hybridized with the first and second target nucleic acid.

Claims 52 and 53, as amended, recite a method of multiplex detection of target nucleic acids where a target nucleic acid is detected by being hybridized with a pool of oligonucleotides

and then being contacted with a substrate comprising a different population of probe oligonucleotides, where the different population of probe oligonucleotides is randomly distributed on the surface of the substrate. None of the cited references teaches the use of a randomly distributed array of oligonucleotides for the capture of a pool of oligonucleotides hybridized to nucleic acid targets.

Beattie et al. teaches the use of ordered arrays for the capture of oligonucleotide-DNA hybrids. In particular, Beattie et al., teaches the use of automated systems for the ordered deposition of oligonucleotides on the substrate

A Microlab 2200 robotic fluid-delivery system ..., supplied with a four-needle delivery head, was used to place submicroliter droplets onto glass slides. The Microlab 2200 system was programmed (using resident software) to deliver droplets of 200 nL onto each slide as previously described. (citation omitted)

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Oligonucleotide probes containing 5'-terminal amino modifications were dissolved in H<sub>2</sub>O to a final concentration of 20uM, and 200nL droplets of each probe were applied to the epoxysilanized glass slides using a Hamilton Microlab 2200 station equipped with a multiprobe head. Rows of three droplets of each probe were attached to observe the reproducibility of the results.

(Beattie et al., col. 19, line 41 - col. 20, line 2.)

Thus, the arrays of in Beattie et al. are composed of ordered arrangements of oligonucleotides.

Likewise, the arrays in Lipshutz et al. are ordered arrangements of oligonucleotides

The nucleic acid templates are all attached to a solid support thereby forming a "template array" (see Fig. 1, "DNA chip"). In a preferred embodiment, each template nucleic acid is located in a particular preselected region on the solid support. Thus, for example the DNA chip of Fig. 1 shows an array of "rectangles" where each rectangle contains a different template nucleic acid.

(Lipshutz et al., col. 10, line 20 – line 26.)

Thus, the references cited by the Examiner fail to teach teaches or suggest all of the elements of the rejected claims. Therefore, Beattie et al. in combination with Lipshutz et al., as evidenced by Sinha et al., cannot render the invention recited in claims 52 and 53 obvious and withdrawal of the rejection is respectfully requested.

**CONCLUSION**

In light of the Amendments and Remarks herein, Applicant submits that the claims are in condition for allowance and respectfully request a notice to this effect. Should the Examiner have any questions, he is invited to call the undersigned attorney.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including extension of time fees, to Deposit Account 500417 and please credit any excess fees to such deposit account.

Respectfully submitted,

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EXHIBIT A    *Syvänen Nature Reviews* 2:930-942 (2001)

# Linkage of IL-6 with Neutrophil Chemoattractant Expression in Virus-Induced Ocular Inflammation

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**PURPOSE.** Herpes simplex virus (HSV)-1 infection of the murine cornea is known to stimulate a vigorous interleukin (IL)-6 response, but whether this pleiotropic cytokine is an essential participant in corneal inflammation is unclear. This study was designed to compare the early inflammatory response in IL-6 gene-deficient mice to that in wild-type hosts.

**METHODS.** Gene knockout and wild-type mice (C57BL/6 background) were infected intracorneally with HSV-1 (strain RE) and observed through clinical examination and immunohistochemistry for the development of corneal opacity. Virus corneal titers were determined by standard plaque assay on Vero cells. Cytokine and chemokine levels in corneal lysates were measured with commercial ELISA kits.

**RESULTS.** Corneal opacity in IL-6<sup>-/-</sup> mice was substantially diminished in comparison with IL-6<sup>+/+</sup> hosts 24 to 48 hours after intracorneal viral infection, and corneal levels of (MIP)-2 and MIP-1 $\alpha$  were significantly reduced. Local administration of IL-6 at the time of infection restored corneal opacity and chemokine levels to that of wild-type hosts. Antibody neutralization of endogenous IL-6 in IL-6<sup>+/+</sup> animals reduced corneal opacity scores and MIP-2 levels to that of IL-6<sup>-/-</sup> mice. Ex vivo studies with excised corneal buttons revealed that uninfected IL-6<sup>-/-</sup> corneas injected with IL-6 produced MIP-2 and MIP-1 $\alpha$  at levels comparable to that seen in IL-6<sup>+/+</sup> hosts.

**CONCLUSIONS.** Collectively, these results suggest that IL-6 promotes corneal inflammation by acting in an autocrine-paracrine fashion to induce resident corneal cells to make MIP-2 and MIP-1 $\alpha$ , which in turn recruit neutrophils to the virus infection site. (*Invest Ophthalmol Vis Sci.* 2002;43:737-743)

Interleukin-6 (IL-6) is a cytokine that is rapidly produced at local tissue sites after disruption of homeostasis due to trauma or infection.<sup>1</sup> It is functionally diverse, influencing the growth, differentiation, and activation of cells and their expression of proteins principally in inflammation, immune responses, and hematopoiesis. To evaluate the inflammatory role of IL-6, studies have been conducted in gene-disrupted mice. It has been found that antigen-induced arthritis is absent or substantially reduced in the IL-6<sup>-/-</sup> mice.<sup>2,3</sup> Such mice are also resistant to experimental autoimmune encephalomyelitis.<sup>4,5</sup> Administration of exogenous IL-6 restores susceptibility.<sup>4</sup> Other studies have shown that less tissue injury is observed in turpentine<sup>6</sup> or carrageenan-treated IL-6<sup>-/-</sup> hosts<sup>7</sup> and that treatment with an anti-IL-6 receptor (IL-6R), mAb is protective

in a murine model of colitis.<sup>8</sup> Thus, IL-6 can be an important promoter of inflammation.

However, IL-6 can also function as an anti-inflammatory cytokine. For example, Xing et al.<sup>9</sup> reported that acute inflammatory responses in animal models of endotoxic lung or endotoxemia were more severe in IL-6<sup>-/-</sup> mice. Administration of recombinant IL-6 reduced circulating levels of proinflammatory mediators. IL-6 can also produce an anti-inflammatory effect by inducing glucocorticoids and natural antagonists of IL-1 $\alpha$  (reviewed in Ref. 10). Additionally, IL-6 may exist as a bystander, neither actively promoting nor inhibiting inflammation.<sup>11</sup>

Ongoing studies in our laboratory have sought to identify and characterize the mediators that participate in the inflammatory cascade in the herpes simplex virus (HSV)-1-infected murine cornea. In this model, the inflammatory response can progress over a 14- to 21-day period to severe stromal keratitis, causing permanent blindness.<sup>12-14</sup> As part of the early warning alarm system, IL-6 appears rapidly after both virus<sup>15</sup> and bacterial infection.<sup>16-18</sup> Indeed, it is one of the more abundantly produced cytokines and can persist as inflammation progresses.

IL-6 is made by a wide variety of cell types, including those found in ocular tissue. We have shown that IL-1 $\alpha$  and TNF- $\alpha$  can induce human corneal fibroblasts and epithelial cells to synthesize and secrete IL-6.<sup>19</sup> Additionally, studies in excised mouse corneas have revealed that IL-6 mRNA induced by endogenous IL-1 $\alpha$  is found in all three resident corneal cell types and that the corneal epithelial layer is the most abundant producer of protein.<sup>20</sup> Kanangat et al.<sup>21</sup> have reported that HSV-1 infection selectively upregulates IL-6 gene expression in the murine epithelial-like cell line EMT-6.

Although IL-6 is quickly produced after HSV-1 corneal infection, it is not known whether it is a necessary component of the inflammatory cascade. We, therefore, initiated studies to investigate whether IL-6 may be involved in the modulation of chemokine expression. We focused on macrophage inflammatory protein (MIP)-2 and MIP-1 $\alpha$ , because these chemokines are known to rapidly recruit neutrophils into the cornea after HSV-1 infection.<sup>22</sup> The experimental approach was to compare the inflammatory response elicited by HSV-1 intracorneal infection in IL-6 gene-deficient mice with that of their wild-type counterparts. In this study, IL-6 was a critical component of the early inflammatory response. Our data indicate that IL-6 enhanced the production of MIP-2 and MIP-1 $\alpha$ , chemokines important in the recruitment of neutrophils into the HSV-1-infected cornea.

## MATERIALS AND METHODS

### Animals

Four-week-old IL-6 gene-deficient mice and matched control mice (C57BL/6 mice) were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with federal, state, and local regulations.

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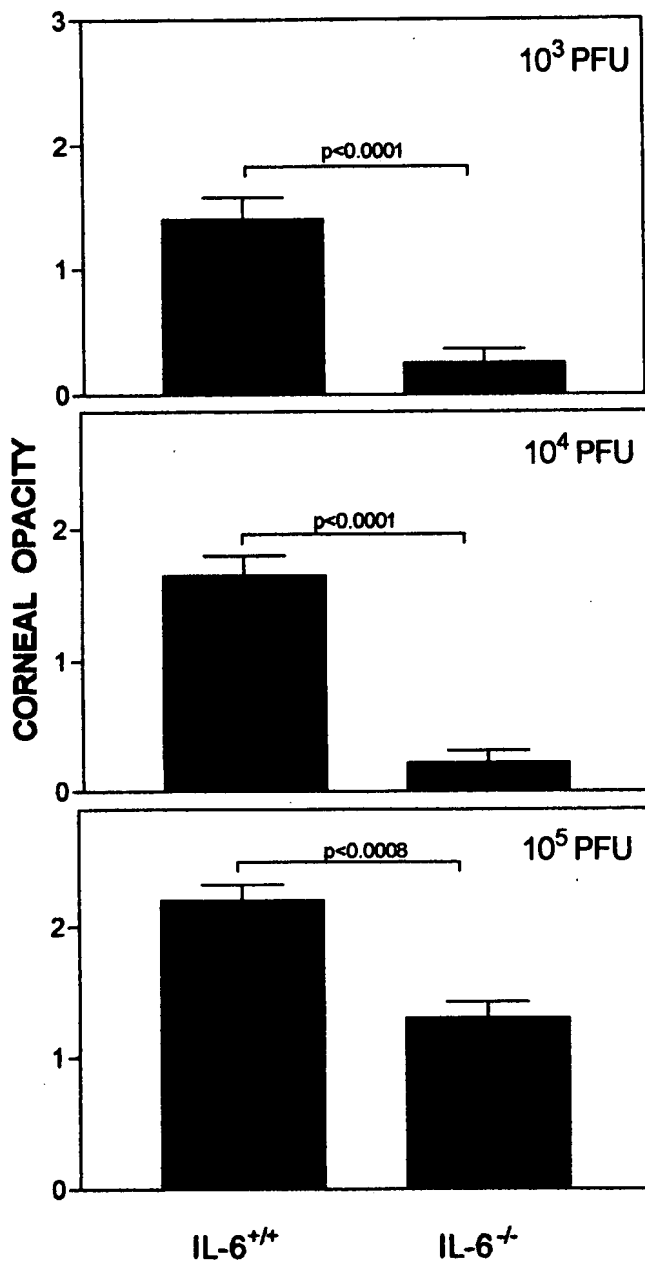


FIGURE 1. IL-6<sup>-/-</sup> mice displayed less severe corneal opacity than IL-6<sup>+/+</sup> mice 48 hours after HSV-1 infection. Hosts were infected intracorneally with the indicated concentrations of HSV-1 and individually scored for corneal disease. Data are the mean  $\pm$  SE of five corneas per group.

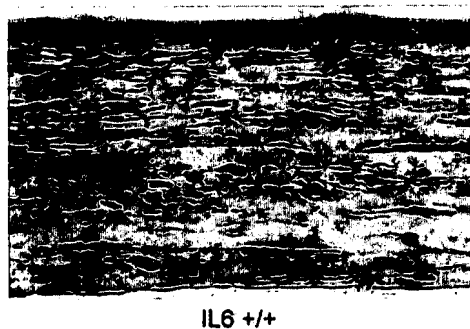


FIGURE 2. Photomicrographs of cross sections of corneas from IL-6<sup>+/+</sup> (left) and IL-6<sup>-/-</sup> (right) mice, removed 48 hours after infection with 10<sup>3</sup> PFU HSV-1 and stained with an mAb RB6-8C5. Note the swelling and extensive leukocyte infiltrate in the IL-6<sup>+/+</sup> cornea. Magnification,  $\times 400$ .

## Antibodies and Reagents

Rat IgG mAb to mouse IL-6 and murine recombinant IL-1 $\alpha$  were purchased from Biosource International (Camarillo, CA); recombinant murine (rm)IL-6 from Endogen, Inc. (Woburn, MA); and hamster IgG monoclonal antibody to mouse IL-1 $\alpha$  from R&D Systems (Minneapolis, MN). Rat RB6-8C5 monoclonal antibody (mAb), a gift from Robert Coffman (DNAX Research Institute, Palo Alto, CA), was prepared as previously described.<sup>23</sup> RB6-8C5 mAb reacts with the Ly-6G antigen, which is expressed on neutrophils and other granulocytes at a level that directly correlates to the differentiation and maturation stage of the cell.

## Intracorneal and Subconjunctival Inoculations

Intracorneal injection was accomplished by first puncturing the corneal epithelium wall with a 30-gauge disposable needle. A 30-cm 32-gauge stainless steel needle attached to a dispenser (Hamilton, Reno, NV) was then threaded into the corneal stroma and 1.0  $\mu$ L of solution containing the appropriate dose of HSV-1 strain RE (10<sup>3</sup>–10<sup>5</sup> plaque-forming units [PFU]) was injected. In some experiments the 1.0- $\mu$ L inoculum contained a mixture of HSV-1 (10<sup>5</sup> PFU/ $\mu$ L) admixed with mAb to IL-6 (5  $\mu$ g/ $\mu$ L) or rmIL-6 (50 ng/ $\mu$ L). These mixtures were prepared immediately before use and directly administered. Pilot studies established that neither addition of IL-6 nor antibody to IL-6 impaired virus infectivity. Subconjunctival antibody injections were performed by using a 2-cm 32-gauge needle and syringe (Hamilton) to penetrate the perivascular region of the conjunctiva and deliver 4  $\mu$ L into the subconjunctival space. To score corneal opacity, eyes were graded on a scale of 0 to +4 by visual observation, using a dissecting biomicroscope. Eyes were graded as follows: 0, clear cornea; +1, slight corneal haze; +2, moderate corneal opacity; +3, severe corneal opacity with visible iris; +4, severe corneal opacity with iris not visible. Eyes were examined in a coded manner, with the reader unaware of the treatment administered.

## Incubation of Corneas Ex Vivo

After intracorneal injection, the corneas were immediately excised, trimmed with a 2-mm trephine, and incubated individually in 200 to 250  $\mu$ L RPMI-1640 medium in a polypropylene tube at 37°C in 5% CO<sub>2</sub> for 10 hours. The medium, with or without, corneas was then frozen at -70°C.

## Chemokine and Cytokine Assays

Samples containing corneas were thawed, minced, and processed for 80 seconds (Tissue Tearer; Biospec Products, Bartlesville, OK), sonicated for 20 seconds, and clarified by centrifugation at 150g for 10 minutes, thus producing clarified corneal lysates. Samples (clarified corneal lysates or supernatant) were assayed by ELISA for MIP-2, MIP-1 $\alpha$ , and IL-6. The MIP-2 kit (assay sensitivity, 1.5 pg/mL), and MIP-1 $\alpha$  kit (assay sensitivity, 1.5 pg/mL), were purchased from R&D Systems (Minneapolis, MN). The IL-6 kit (assay sensitivity, 7.0 pg/mL) were purchased from Endogen, Inc.

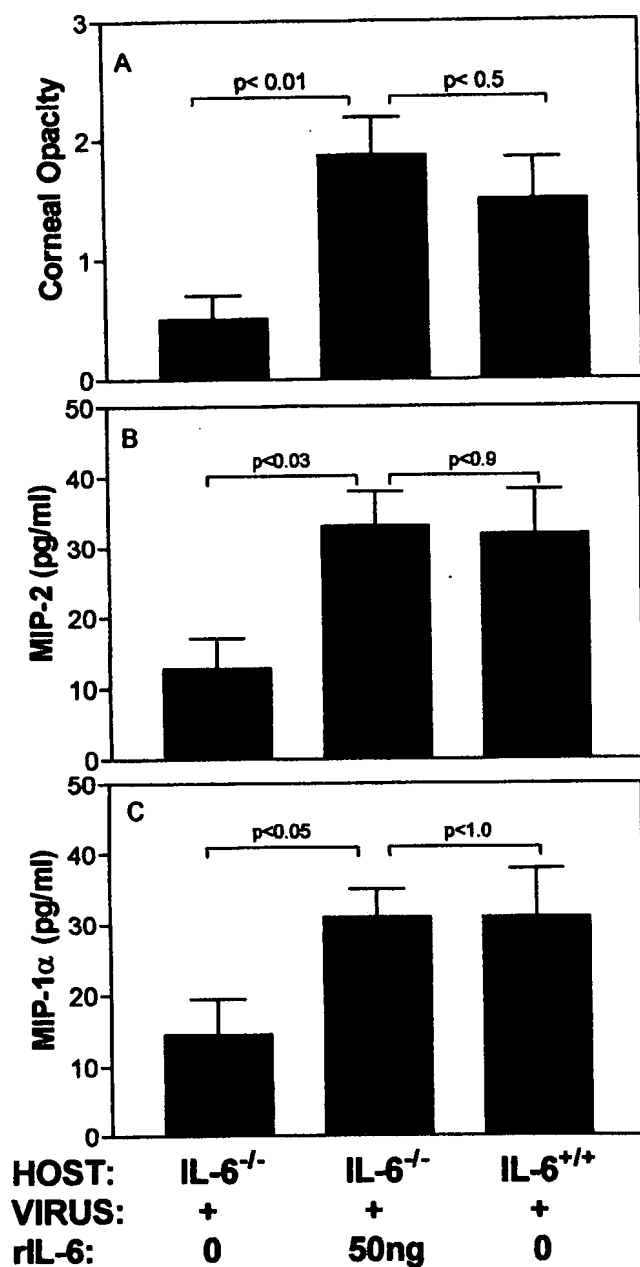


FIGURE 3. Effect of IL-6 treatment on (A) corneal opacity and (B) MIP-2 and (C) MIP-1α levels in HSV-1-infected *IL-6*<sup>-/-</sup> corneas. IL-6 was mixed with 10<sup>4</sup> PFU HSV-1 and inoculated intracorneally into *IL-6*<sup>-/-</sup> mice. Additional *IL-6*<sup>-/-</sup> and *IL-6*<sup>+/+</sup> groups were treated with virus only. At 48 hours after infection, corneal opacity was scored. Corneal lysates were then prepared and assayed for chemokines by ELISA. Data are the mean ± SE of five corneas per group.

### Immunohistochemistry

Immunohistochemical staining was performed using a slight modification of the procedure of Hendricks et al.<sup>24</sup> Infected eyes were enucleated and embedded in optimal cutting temperature compound (Tissue Tek; Sakura Finetek, Torrance, CA) and 6-μm sections were cut at -20°C. The sections were fixed in cold acetone for 10 minutes and then blocked with normal goat serum. The primary antibody, RB6-8C5 (5 μg/mL) was applied to the tissue for 1 hour at room temperature. The sections were then washed and stained using the streptavidin-biotin complex immunoperoxidase staining procedure, according to the manufacturer's protocol (Zymed Laboratories, South San Francisco, CA). The slides were washed in distilled water and counterstained in Harris hematoxylin for 3 minutes. The slides were then examined

under a light microscope, and pictures were taken (BX50 camera; Olympus Optical Co., Tokyo, Japan).

### Neutrophil Isolation and Stimulation

Bone marrow (BM) was flushed from the femur and tibia with media (RPMI-1640 with 5% newborn calf serum) using a syringe and 25-gauge needle. The BM cells were centrifuged (1200 rpm for 5 minutes), washed twice, layered over a gradient (Histopaque 1119 and 1077; Sigma, St. Louis, MO), and centrifuged at 700g for 30 minutes. The enriched neutrophil layer was removed, washed twice in medium and treated with red blood cell lysis buffer (Sigma). Contaminating monocytes were depleted by adherence (30 minutes at 37°C) to a polystyrene tissue culture plate<sup>25</sup> (Corning Glass Co., Corning, NY). Neutrophil purity was consistently more than 99%, as assessed by staining (HEMA 3; Biochemical Sciences Inc., Swedesboro, NJ) of cytospin slides (Shandon, Pittsburgh, PA).

For stimulation 1 × 10<sup>6</sup> neutrophils in 0.5 mL medium were placed in triplicate in 24-well tissue culture plates (Corning Glass Co.). The wells were precoated with 0.5 mL newborn calf serum per well for 1 hour at 37°C and then washed three times with PBS. Stimulations were performed by the addition of rMIL-6 to wells at a final concentration of 5 or 50 ng/mL. rMIL-1α was used at 1 or 10 ng/mL. Phorbol 12-myristate, 13 acetate (PMA; 30 ng/mL) served as the positive control and medium only as the negative control. After incubation for 8 hours at 37°C in 5% CO<sub>2</sub> supernatants were removed, clarified by centrifugation, and assayed for chemokine content by ELISA.

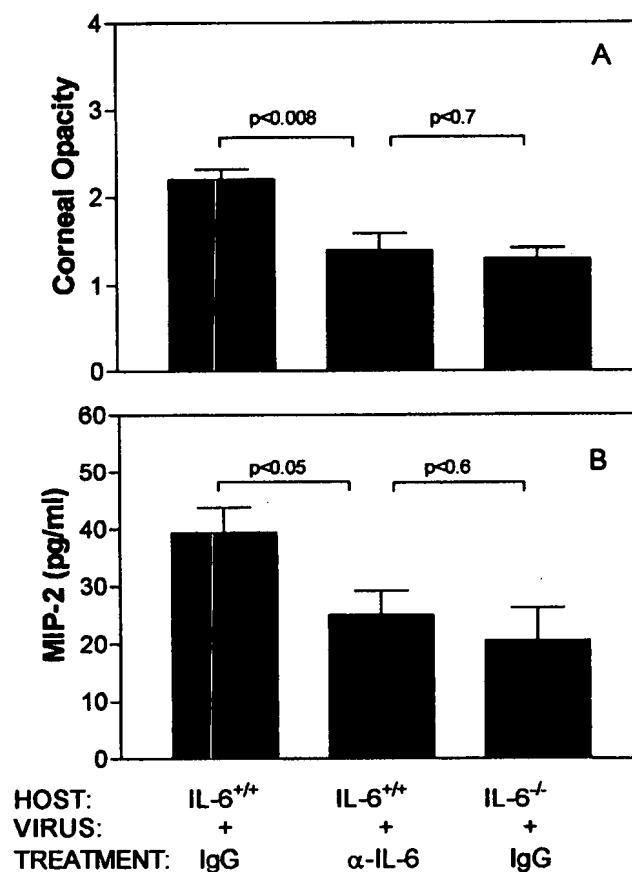
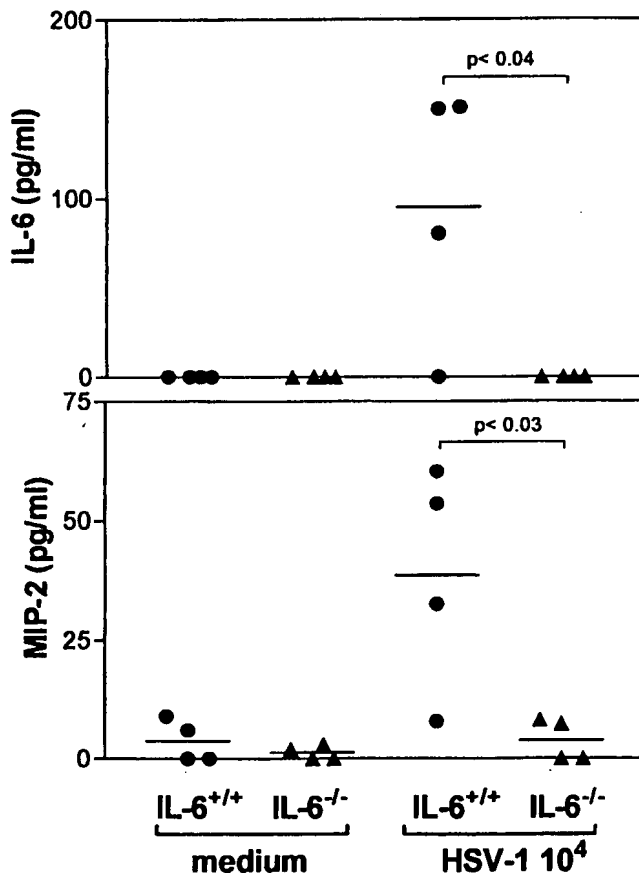


FIGURE 4. Antibody neutralization of IL-6 suppressed (A) corneal opacity and (B) MIP-2 levels in *IL-6*<sup>+/+</sup> mice. Wild-type mice were infected intracorneally with 10<sup>5</sup> PFU HSV-1, admixed with neutralizing mAb to IL-6 (5 μg/μL). Additional antibody (4 μL) was administered subconjunctivally at 12, 24, and 36 hours after infection. Other *IL-6*<sup>+/+</sup> and *IL-6*<sup>-/-</sup> hosts received control rat IgG. Corneal opacity was recorded at 48 hours, and corneal lysates were assayed for MIP-2.



**FIGURE 5.** Cytokine (*top*) and chemokine (*bottom*) responses in HSV-1-infected corneas of IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> hosts ex vivo. Immediately after HSV-1 infection ( $10^4$  PFU intracorneally), corneas from IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> mice were excised and incubated in vitro for 10 hours. Supernatants were then assayed for the indicated mediators by ELISA. Bars depict the mean in each group.

### Statistical Analysis

The Mann-Whitney test was used to determine significant differences in the corneal opacity scores between treated and control groups. Student's *t*-test was used to evaluate mediator responses between treated and control cells. The level of confidence at which the results were judged significant was  $P < 0.05$ .

### RESULTS

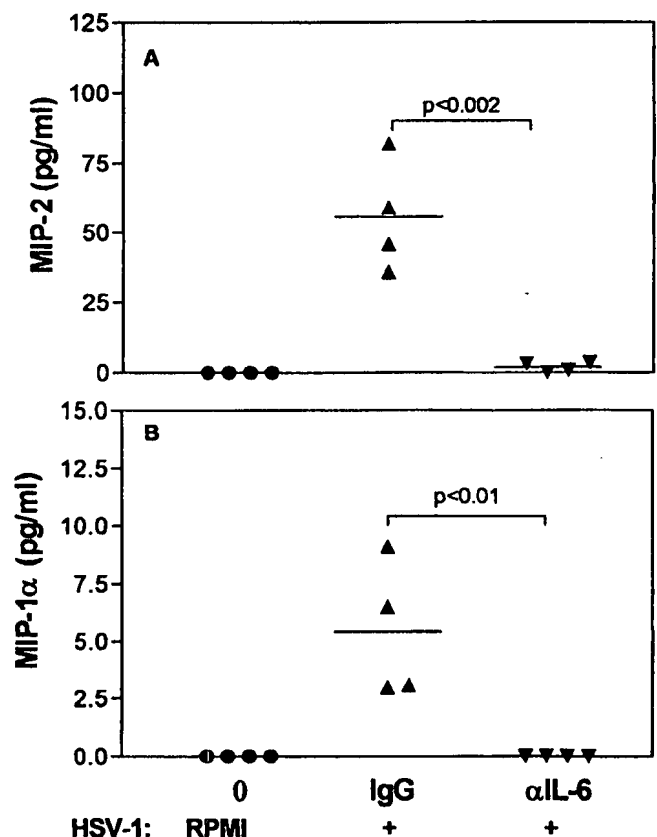
#### HSV-1-Induced Corneal Opacity in IL-6<sup>-/-</sup> Mice

When HSV-1 is inoculated intracorneally a rapid inflammatory response is elicited, which is evident by 24 hours and peaks at 48 hours after infection.<sup>22</sup> We investigated whether disruption of the IL-6 gene would influence this initial response. It was found that IL-6<sup>-/-</sup> animals challenged with virus over a 100-fold dose range consistently showed significantly less corneal cloudiness than did IL-6<sup>+/+</sup> mice, as determined by microscopic observation during the first 2 days postinfection. Figure 1 shows representative results from one of five independent experiments. This striking disparity was confirmed histologically: IL-6<sup>-/-</sup> corneas were less swollen and contained many fewer neutrophils positively stained with RB6-8C5 mAb than did corneas from IL-6<sup>+/+</sup> hosts (Fig. 2). The difference in inflammation was transient; the opacity scores of the two groups were no longer significantly different 4 days after infection. The distinct disparity in corneal responsiveness observed during the first 2 days could not be attributed to a

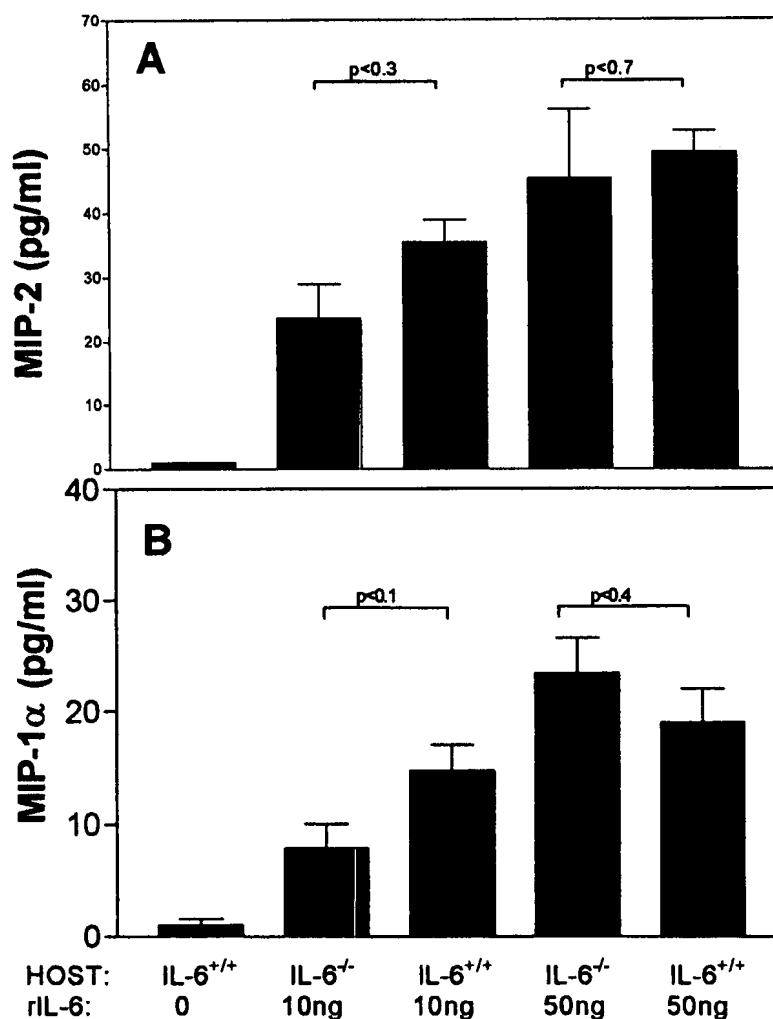
difference in HSV-1 replication as the titers of infectious virus in the wild-type mice ( $1.0 \times 10^5 \pm 0.4 \times 10^5$ ) were not significantly different ( $P > 0.7$ ) from that observed in the ocular tissue of knockout hosts ( $1.2 \times 10^5 \pm 0.5 \times 10^5$ ). Similar virus titer results were obtained in two additional independent experiments.

#### Effect of IL-6 Administration on Corneal Opacity and Chemokine Expression

To evaluate whether exogenous IL-6 could restore heightened corneal opacity, reconstitution experiments were performed in IL-6<sup>-/-</sup> mice. It was found that a single inoculation of IL-6 administered locally, together with the virus challenge inoculum restored corneal opacity to the levels seen in IL-6<sup>+/+</sup> mice at 24 and 48 hours after infection (Fig. 3A; only 48-hour results from a representative experiment are shown). No corneal cloudiness was observed in animals given 50 ng IL-6 in the absence of HSV-1. Administration of IL-6 together with the virus significantly raised the levels of chemokines MIP-2 (Fig. 3B) and MIP-1 $\alpha$  (Fig. 3C) relative to that seen in control mice treated with virus only. Furthermore, the amounts of each mediator were comparable to the levels found in the corneas of the IL-6<sup>+/+</sup> hosts and thus correlated with increased mean corneal opacity scores.



**FIGURE 6.** (A) MIP-2 and (B) MIP-1 $\alpha$  responses in HSV-1-infected IL-6<sup>+/+</sup> corneas treated with anti-IL-6 ex vivo. HSV-1 ( $10^4$  PFU) plus neutralizing antibody to IL-6 (2.5  $\mu$ g) or control IgG were injected intracorneally in two groups. A third group was given a placebo. Corneas were immediately excised and individually incubated in vitro for 10 hours. Corneal lysate was then assayed for the indicated mediators. Each symbol represents the mediator value for an individual mouse, with the bar representing the mean levels in the group.



**FIGURE 7.** Chemokine response in IL-6-treated uninfected IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> corneas ex vivo. Immediately after IL-6 was administered intracorneally at the doses shown, corneas from IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> mice were excised and incubated in vitro for 10 hours. Corneal lysates were then prepared and assayed for (A) MIP-2 and (B) MIP-1α. Data are the mean ± SE of three corneas per group.

#### Effect of IL-6 Neutralizing Antibody on Corneal Opacity and MIP-2 Response in HSV-1-Infected IL-6<sup>+/+</sup> Hosts

We next determined whether antibody neutralization of endogenous IL-6 would influence HSV-1 induced corneal opacity in IL-6<sup>+/+</sup> mice. IL-6 antibody was introduced intracorneally at a concentration of 2.5 μg/μL when admixed with the challenge virus. In preliminary studies, it was determined that a concentration of anti-IL-6 antibody lower than 2.5 μg/μL was not sufficient to neutralize the effect of IL-6 (data not shown). Additional antibody was administered subconjunctivally at 12-hour intervals, and corneas were examined at 48 hours after infection. Figure 4A shows results from a representative experiment in which corneal opacity scores in IL-6<sup>+/+</sup> hosts after anti-IL-6 antibody treatment were significantly reduced compared with IgG-treated control animals and indeed were comparable to that seen in IL-6<sup>-/-</sup> mice. In addition, corneal MIP-2 concentrations were also significantly reduced to levels observed in IL-6<sup>-/-</sup> mice (Fig. 4B).

#### Induction of MIP-2 and MIP-1α in Infected and Uninfected Corneal Tissue

We postulated that HSV-1 infection induces resident corneal cells to synthesize and secrete IL-6. This hypothesis was investigated using excised corneas to avoid production of mediators by infiltrating leukocytes. Figure 5 shows that after HSV-1 infection, corneal tissue from IL-6<sup>+/+</sup> hosts had high levels of IL-6, whereas none was seen in virus-infected IL-6<sup>-/-</sup> corneas.

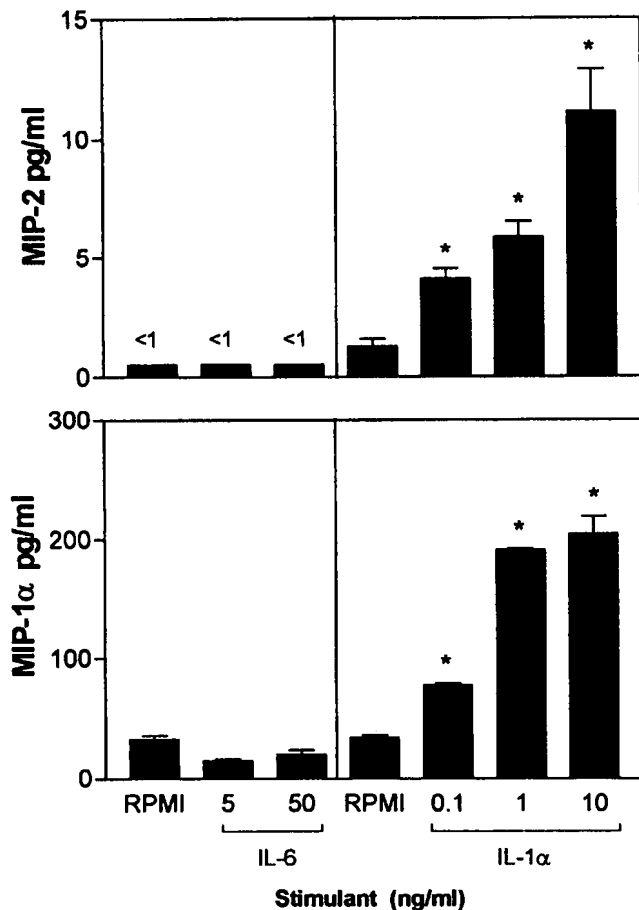
Significantly, MIP-2 levels in infected IL-6<sup>+/+</sup> corneas were 10-fold higher than that seen in infected corneas without a functional IL-6 gene. This suggests that IL-6 induces resident corneal cells to make a neutrophil chemoattractant. Support for this prediction comes from experiments in which antibody neutralization of IL-6 in HSV-1-infected IL-6<sup>+/+</sup> corneas abrogated production of MIP-2 and MIP-1α (Fig. 6).

Additionally we found that administration of exogenous IL-6 to uninfected IL-6<sup>-/-</sup> corneal tissue at a 10- to 50-ng dose stimulated a 24- to 45-fold increase in MIP-2 (Fig. 7A) and an 8- to 23-fold increase in MIP-1α (Fig. 7B). These amounts were comparable to that stimulated by IL-6 in uninfected wild-type corneas.

#### Effect of IL-6 on MIP-2 and MIP-1α Induction in Neutrophils

The IL-6 produced by resident corneal cells could also induce neutrophils recruited to the HSV-1 infection site to produce chemokines. Accordingly, we tested whether IL-6 stimulates MIP-2 or MIP-1α synthesis in these cells. Figure 8 depicts representative data from one of four independent experiments. Five or 50 ng/mL IL-6 added to bone marrow-derived neutrophils did not enhance production of MIP-2 or MIP-1α above background levels (a less than twofold increase). In contrast, IL-1α stimulated a two- to sixfold increase in MIP-1α. MIP-2 production was also enhanced by IL-1α in a dose-dependent manner, although the amounts generated were substantially lower (18- to 31-fold) than MIP-1α. Neutrophils stimulated with





**FIGURE 8.** Neutrophils exposed to IL-1 $\alpha$  but not IL-6 produce (top) MIP-2 and (bottom) MIP-1 $\alpha$ . Bone marrow-derived neutrophils ( $10^6$ /well) were prepared and stimulated in triplicate, with the indicated concentrations of IL-6 and IL-1 $\alpha$  for 8 hours. Supernatants were then assayed for chemokines by ELISA. \*Significantly higher than the medium control ( $P < 0.001$ ).

30 ng/mL PMA produced on average 209 pg/mL MIP-2 and 2198 pg/mL MIP-1 $\alpha$ . Incubation of neutrophils with both IL-6 and MIP-1 $\alpha$  did not elevate chemokine production above that seen with IL-1 $\alpha$  alone.

IL-6 failed to stimulate neutrophils purified from the bone marrow of IL-6 $^{-/-}$  mice to produce MIP-2 or MIP-1 $\alpha$  when tested as described. However, IL-1 $\alpha$  at 10 ng/mL induced a six- and ninefold increase in MIP-2 and MIP-1 $\alpha$ , respectively, over background levels. The inability of IL-6 to induce chemokine production by neutrophils was also observed when the incubation period was extended from 10 to 18 hours.

## DISCUSSION

In this study, evidence showed IL-6 played a prominent role in initiating corneal inflammation after HSV-1 infection. Mice with a disrupted IL-6 gene displayed substantially reduced corneal opacity 24 to 48 hours after intracorneal infection in comparison with their wild-type counterparts, as judged both clinically and histologically. A single 50-ng dose of IL-6 administered locally at the time of infection restored corneal disease to wild-type levels. Furthermore, antibody neutralization of endogenous IL-6 in IL-6 $^{+/+}$  hosts reduced corneal inflammation to that observed in IL-6 $^{-/-}$  animals. The recent report that IL-6 abolishes immune privilege in eyes with endotoxin-induced uveitis<sup>26</sup> also supports the view that IL-6 is proinflammatory in ocular tissue.

There are several mechanisms by which HSV-1 infection could induce IL-6. One is that virus infection may directly activate IL-6 gene expression in corneal cells.<sup>21</sup> Alternatively, virus infection may induce IL-1 $\alpha$ , which in turn induces IL-6. This latter possibility is supported by earlier studies that showed that synthesis of IL-6 stimulated by mechanical trauma in excised corneal buttons was abrogated by antibody to IL-1 $\alpha$  but not antibodies to IL-1 $\beta$  or TNF- $\alpha$ .<sup>20</sup>

Our present data provide evidence that IL-6 promoted induction of neutrophil chemoattractants. Initial evidence for this conclusion comes from *in vivo* studies that show that IL-6 administered to infected corneas of IL-6 $^{-/-}$  mice elevates MIP-2 and MIP-1 $\alpha$  to wild-type levels and that antibody neutralization of IL-6 locally in wild-type corneas reduces MIP-2 to IL-6 $^{-/-}$  levels. It is worth noting that our follow-up *ex vivo* studies demonstrated that uninfected as well as infected resident cells in corneal tissue exposed to IL-6 produced MIP-2 and MIP-1 $\alpha$ . Thus, IL-6 induction of chemokines does not require that the corneal cells be infected. Collectively, a likely scenario based on our findings is that HSV-1 corneal infection initiates an inflammatory cascade in which IL-1 $\alpha$  induces IL-6 and then these two cytokines through autocrine-paracrine action stimulate resident corneal cells to produce neutrophil chemoattractants. An earlier report showing that IL-1 $\alpha$  inoculated into the mouse cornea results in the production of MIP-2<sup>22</sup> is compatible with this conclusion.

In contrast to resident corneal cells, exposure of purified mouse neutrophils to recombinant IL-6 caused little or no elevation of MIP-2 or MIP-1 $\alpha$ . However, IL-1 $\alpha$  induced substantial levels of MIP-1 $\alpha$  and more modest but significant amounts of MIP-2. This suggests that resident cells in the cornea are the principal targets of IL-6, whereas IL-1 $\alpha$  acts on both resident corneal cells and infiltrating leukocytes. Whether IL-6 can induce infected corneal cells or infiltrating neutrophils to make and secrete other proinflammatory mediators remains to be determined. Additionally, IL-6 may also influence the effector responses of neutrophils.<sup>27</sup>

It has been reported that IL-10 is produced constitutively in the mouse cornea and acts to antagonize development of virus-induced inflammation.<sup>28</sup> It may be speculated that IL-6 enhances corneal disease because it suppresses endogenous IL-10 expression. However, in three independent experiments we found that administration of IL-6 to IL-6 $^{-/-}$  mice did not alter IL-10 corneal levels (Fenton and Lausch, unpublished observations, 2001). In contrast, antibody neutralization of endogenous IL-10 significantly enhanced production of IL-6, MIP-2, and MIP-1 $\alpha$ .<sup>28</sup> We conclude that HSV-1 infection of IL-6-deficient mice did not result in upregulation of the anti-inflammatory cytokine IL-10 as was seen in *Candida albicans* infection.<sup>29</sup>

IL-6 $^{-/-}$  mice have been observed to produce a lower antibody response or a reduced cytotoxic T-lymphocyte response to certain selected viral pathogens<sup>30</sup> but not others.<sup>31</sup> We found that although HSV-1 ocular titers in IL-6 $^{-/-}$  mice were similar to those of IL-6 $^{+/+}$  hosts at 48 hours after infection 8 of 15 (53%) showed development of fatal encephalitis, whereas only 1 of 15 (7%) IL-6 $^{+/+}$  mice died. These results are in agreement with the report of LeBlanc et al.<sup>32</sup> Neutrophils and mononuclear cells are known to help limit HSV-1 growth and spread to the central nervous system after ocular infection.<sup>23,33</sup> Their diminished early recruitment due to reduced MIP-2 and MIP-1 $\alpha$  production would be expected to increase IL-6 $^{-/-}$  host susceptibility to HSV-1. Our results may also help to explain the impaired neutrophil recruitment and increased susceptibility seen in IL-6 $^{-/-}$  mice infected with *Listeria monocytogenes*<sup>34</sup> and *C. albicans*.<sup>29</sup> In addition, or alternatively, the stronger inflammatory response in the IL-6 $^{+/+}$  cornea may generate a cytokine milieu that favors a greater, and thus more protective, T helper cell-1 response.<sup>35,36</sup>

In summary, our findings showed IL-6 to be an important participant in the cytokine cascade triggered by HSV-1 corneal infection. By inducing neutrophil chemoattractants IL-6 amplified the inflammatory response. Our data are in agreement with the report of Smith et al.,<sup>37</sup> wherein IL-6 was associated with MIP-1 $\alpha$  expression in a bleomycin-induced lung injury model. Also, Romano et al.<sup>35</sup> observed that IL-6<sup>-/-</sup> mice exhibit reduced chemokine production and defective leukocyte recruitment in subcutaneous air pouches injected with inflammatory stimuli. This defect could be reversed by IL-6 administration. In follow-up studies IL-6 itself did not induce human endothelial cells, which have no IL-6R, to make chemokine, but IL-6-soluble (s)IL-6R complexes were active. Collectively, these studies and ours indicate that chemokine production can be induced in distinctly different cell types by IL-6 per se, or by IL-6-sIL-6R complexes. This suggests that IL-6 in one form or another may promote chemokine expression in a variety of tissues. Thus, IL-6 represents a potential therapeutic target for suppressing ocular inflammation.

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## ACCESSING GENETIC VARIATION: GENOTYPING SINGLE NUCLEOTIDE POLYMORPHISMS

*Ann-Christine Syvänen*

Understanding the relationship between genetic variation and biological function on a genomic scale is expected to provide fundamental new insights into the biology, evolution and pathophysiology of humans and other species. The hope that single nucleotide polymorphisms (SNPs) will allow genes that underlie complex disease to be identified, together with progress in identifying large sets of SNPs, are the driving forces behind intense efforts to establish the technology for large-scale analysis of SNPs. New genotyping methods that are high throughput, accurate and cheap are urgently needed for gaining full access to the abundant genetic variation of organisms.

**LINKAGE DISEQUILIBRIUM  
MAPPING**  
Analysing single nucleotide  
polymorphism alleles in  
population-based studies to  
identify loci that are associated  
with a particular disease or  
phenotype.

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Comparison of genomic DNA sequences in different individuals reveals some positions at which two, or in some cases more than two, bases can occur. These single nucleotide polymorphisms (SNPs) are highly abundant, and are estimated to occur at 1 out of every 1,000 bases in the human genome<sup>1,2</sup>. Depending on where a SNP occurs, it might have different consequences at the phenotypic level. SNPs in the coding regions of genes that alter the function or structure of the encoded proteins are a necessary and sufficient cause of most of the known recessively or dominantly inherited monogenic disorders. These SNPs are routinely analysed for diagnostic purposes. Another important group of SNPs are those that alter the primary structure of a protein involved in drug metabolism. These SNPs are targets for pharmacogenetic analyses<sup>3</sup>. Missense SNPs in the coding regions of genes, such as the two SNPs in the apolipoprotein E gene<sup>4</sup> and the factor V Leiden mutation<sup>5</sup>, can also contribute to common disease. This type of SNP can be analysed to assess the risk of an individual for a particular disease. In addition, it is likely that SNPs in the regulatory regions of genes might influence the risk of common disease. However, most SNPs are located in non-coding regions of the genome, and have no direct known impact on the phenotype of an individual.

These SNPs are useful as markers in population genetics and evolutionary studies<sup>6,7</sup>.

The reason for the current enormous interest in SNPs is the hope that they could be used as markers to identify genes that predispose individuals to common, multifactorial disorders by using LINKAGE DISEQUILIBRIUM (LD) MAPPING<sup>8,9</sup>. The rationale would be to genotype a collection of SNPs that occur at regular intervals and cover the whole genome to detect genomic regions in which the frequencies of the SNP alleles differ between patients and controls. It is assumed that the SNP alleles are inherited together with the disease-predisposing alleles through the generations because they are physically close to each other. The disease-predisposing genes could then be localized and isolated, and proteins encoded by them would be valuable targets for developing new therapeutic drugs. As a result of the efforts of the SNP Consortium — a collaboration of 14 major pharmaceutical companies and the Wellcome Trust, as well as members of the Human Genome Project<sup>1</sup> — there are almost 2 million SNPs in public databases, and perhaps twice that number of SNPs in commercial databases, such as that of Celera Genomics<sup>2</sup>. The SNPs are now being verified experimentally, and their distributions between and within populations are being assessed<sup>10,11</sup>.

Table 1 | Recent or large studies involving SNP genotyping

Purpose of study	No. of SNPs	No. of samples	No. of genotypes	Method
SNPs in the <i>ACE</i> gene <sup>131</sup>	13	1,300	17,000	Restriction-site analysis
<i>NOD2</i> gene in Crohn disease <sup>132</sup>	13	1,300	17,000	Invader <sup>115</sup> and TaqMan <sup>50</sup> assays
Mapping complex disease traits in mice <sup>34</sup>	109	299	33,000	Allele-specific PCR <sup>60</sup>
Sequence diversity of the <i>APOE</i> gene <sup>133</sup>	20	2,200	44,000	Oligonucleotide ligation assay <sup>95</sup>
<i>APOE</i> haplotypes in Alzheimer disease <sup>32</sup>	60	1,000	60,000	TaqMan assay <sup>50</sup>
Ancestral alleles of human SNPs <sup>7</sup>	214	412	99,000	GeneChip <sup>39</sup>
Carrier frequencies of disease mutations <sup>134</sup>	31	4,400	140,000	Primer extension on microarrays <sup>82</sup>
Haplotype tagging in diabetes candidate genes <sup>135</sup>	122	1,500	180,000	Invader PCR assay <sup>115</sup>
Linkage disequilibrium in the 5q31 region <sup>136</sup>	312	681	210,000	Multiplex primer extension <sup>69,71,137</sup>

*ACE*, angiotensin I-converting enzyme (peptidyl-dipeptidase A)1; *APOE*, apolipoprotein E; SNP, single nucleotide polymorphism.

But how many SNPs are needed? Although recent studies indicate that LD is structured into discrete blocks in the human genome<sup>12,13</sup>, the range and distribution of LD in different populations is largely unknown. Furthermore, the effect of genotype on disease phenotype varies between disorders and populations owing to genetic and environmental heterogeneity<sup>14</sup>. For these reasons, it is difficult to estimate the number of SNPs or the number of samples that would be required for a successful genome-wide LD study. The estimates that have been made vary widely, from 1 million<sup>15</sup> or 0.5 million<sup>16</sup> to as little as 30,000 SNPs<sup>17</sup>. If 0.5 million SNPs were to be analysed in, for example, 1,000 individuals, and if the project was to be carried out in a year, ~1.5 million SNP genotypes per day would be produced. At genotyping costs as low as US 10 cents per SNP, this example project would cost US \$50 million. The throughput required for this project, which comprises only a modest number of samples, is about 100-fold greater than the capacity of SNP-genotyping technology available now. Both the costs and the throughput would prohibit executing the project in even the largest genotyping centres. For typing 30,000 SNPs in 1,000 individuals in a year, about a tenfold increase in the capabilities of current technology would be enough.

A more feasible alternative to random whole-genome SNP mapping is to use SNP markers in or close to candidate genes, or in candidate genomic regions. For example, the genotyping of 20 SNPs in 50 candidate genes (1,000 SNPs) or of 1 SNP per 10 kb in a 10-million-bp candidate genomic region in 1,000 samples represents a reasonable goal of 1 million genotypes. It would be possible to carry out a project of this size within a few months in an intermediate-sized genotyping centre equipped with modern SNP-genotyping technology. For diagnosis and carrier screening of monogenic disorders and for routine pharmacogenetic applications, tens of SNPs are typically analysed in several thousand samples<sup>3,18,19</sup>. SNP-genotyping technology with acceptable throughput and cost for

these routine applications is now available<sup>20</sup>, although these fields would obviously benefit from lower genotyping costs. TABLE 1 gives some examples of recent or large studies in which SNP genotyping had a fundamental role. The scale of these projects is modest, and it is certain that larger studies that have not been published have been conducted, or are in progress, in the commercial sector.

Key targets to improve SNP genotyping technology are cost, simplicity of assay design, throughput and accuracy. One approach for increasing throughput is to devise automated assay formats of the generally used reaction principles for genotyping individual SNPs, and to use a 'brute force' strategy by multiplying these automated platforms. This strategy is analogous to the implementation of Sanger's dideoxy method for DNA sequencing<sup>21</sup> by the Human Genome Project<sup>22</sup>. A second approach to increase throughput is to multiplex the biochemical genotyping reactions, instead of the platforms. Microarray technology is a typical example of this approach. The most challenging approach is to develop completely new molecular strategies for SNP genotyping. In this article, I review the current state of the art of SNP-genotyping technology, with an emphasis on how amenable the different methods are to multiplexing to increase throughput and bring down the costs of the assays. Several promising new principles and assay formats also discussed.

#### Principles of SNP-genotyping methods

The ability of hybridization with allele-specific oligonucleotides (ASO) to detect a single base mismatch was first shown in 1979 (REF. 23), and then used to detect the sickle-cell mutation in the  $\beta$ -globin gene by Southern blot hybridization to human genomic DNA in 1983 (REF. 24). Identification of a single base change in the  $6 \times 10^9$  bp of the diploid human genome is, however, a demanding task. Not until the PCR technique was invented<sup>25,26</sup>, did it become possible to design useful assays for genotyping SNPs in complex

## REVERSE DOT BLOT

A genotyping method based on hybridization between allele-specific oligonucleotide probes that have been immobilized on a membrane, and amplified DNA fragments in solution.

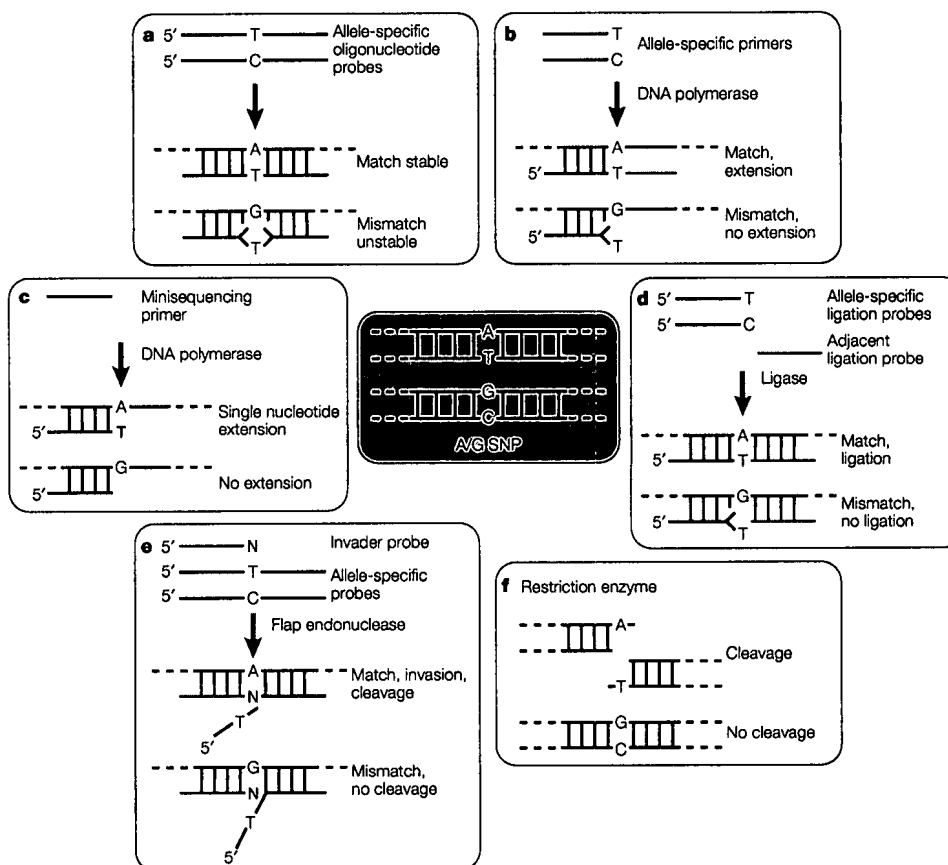
genomes. BOX 1 summarizes six central biochemical reaction principles that underlie SNP-genotyping methods, and FIG. 1 illustrates how some of the current SNP-genotyping platforms have been devised by combining a reaction principle with an assay format and a detection strategy.

Some of the early SNP-genotyping assays used for PCR products were based on ASO hybridization in dot blot<sup>27</sup> or REVERSE DOT BLOT<sup>28</sup> formats. The reverse dot blot format can be viewed as the precursor of the high-density microarray-based methods for multiplex genotyping of SNPs by ASO hybridization<sup>29,30</sup>. Before long, the

PCR technique was developed further by several groups to allow allele-specific amplification and genotyping of SNPs<sup>31</sup>. The use of ASOs as hybridization probes or as PCR primers is the basis for the SNP-genotyping assays that are referred to as 'homogeneous', because they contain no separation steps and are monitored in real time during PCR. These assays are frequently used for large-scale genotyping of SNPs today<sup>32-34</sup>. The solid-phase assays for enzyme-assisted genotyping, using a DNA ligase<sup>35</sup> or a DNA polymerase<sup>36</sup>, were also introduced more than a decade ago. Because the enzyme-assisted methods have proven

## Box 1 | Biochemical reaction principles underlying SNP genotyping

The panels illustrate detection of the A-allele of an A-to-G transition. The G-allele would be detected analogously in a parallel reaction. In panel a, hybridization with allele-specific oligonucleotides (ASOs) is shown. Two short ASO probes are used, usually with the nucleotide complementary to the allelic variant of the single nucleotide polymorphism (SNP) in the middle position of the probe sequence. The probes are allowed to base pair with the target DNA that contains the SNP at conditions in which only perfectly matched probe-target hybrids are stable, and hybrids that contain a mismatch are unstable. In panel b, allele-specific primer extension is shown. Two primers that anneal to their target sequence adjacent to the SNP and have the nucleotide complementary to the allelic variant at their 3'-end are used in primer extension reactions catalysed by a DNA polymerase. Only primers with perfectly



primers with perfectly matched 3'-ends will be extended. In panel c, 'minisequencing' single nucleotide primer extension is shown. One primer that anneals to its target sequence immediately adjacent to the SNP is extended by a DNA polymerase with a single nucleotide that is complementary to the nucleotide at the site of the SNP. The identity of the nucleotide by which the primer becomes extended defines the genotype. In panel d, oligonucleotide ligation is shown. Pairs of oligonucleotide probes that anneal to their target sequence adjacent to each other and have an allele-specific 3'- or 5'-nucleotide at the junction between the probes are used. When the probes are perfectly matched to their target sequence, they will be joined by a ligase, whereas a mismatch at the junction inhibits ligation. In panel e, invasive cleavage is shown. Pairs of allele-specific oligonucleotide probes are used, but the sequence 5' of the SNP is unrelated to the target. In addition, an upstream (invader) oligonucleotide is used that is complementary to the sequence 5' of the SNP. When the allele-specific oligonucleotide is perfectly matched to its target, it is displaced at the SNP site by the upstream invader oligonucleotide, and the formed structure is specifically recognized and cleaved by a FLAP endonuclease, which releases the 5'-part of the probe. In panel f, restriction site cleavage is shown. Restriction endonucleases are used for allele-specific cleavage of the target DNA when a SNP alters the recognition sequence for the enzyme. Target molecules with intact recognition sites will be cleaved, whereas target molecules with altered sites remain uncleaved.

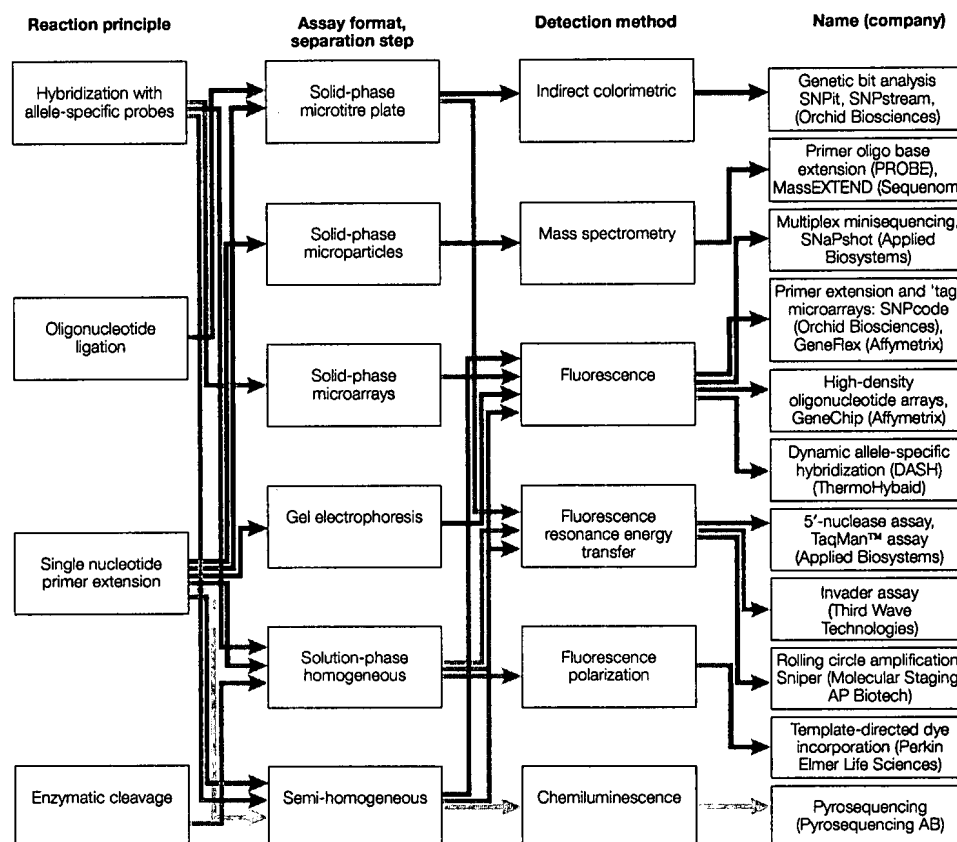


Figure 1 | 'Modular' design of some of the assays for SNP genotyping. Coloured arrows are used to show the reaction principles, assay format and detection methods that make up a particular genotyping method. For example, the TaqMan™ assay involves hybridization with allele-specific oligonucleotides, a solution-phase assay and detection by fluorescence resonance energy transfer. The figure illustrates principles for assay design, and the list of assays is not intended to be comprehensive.

to be more robust and to provide more specific allele distinction than ASO hybridization<sup>37</sup>, these methods have been multiplexed, automated and adapted to various detection strategies, and they provide most of the current high-throughput SNP-genotyping platforms.

All methods used for genotyping SNPs in large diploid genomes depend on PCR amplification of the genomic regions that span the SNPs before the actual genotyping reaction. The PCR provides the required sensitivity and specificity for distinguishing between heterozygous and homozygous SNP genotypes in large, complex genomes. The difficulty of designing and carrying out multiplex PCR reactions is an important factor that limits the throughput of the current SNP-genotyping assays. In the following sections, these methods are discussed in more detail. TABLE 2 summarizes some of their features.

#### Hybridization methods

The thermal stability of a hybrid between an ASO probe and its SNP-containing target sequence is not only determined by the stringency of the reaction conditions, but also by the nucleotide sequence that flanks the SNP

and the secondary structure of the target sequence<sup>24,38</sup> (BOX 1). However, it is difficult to predict, *a priori*, the reaction conditions or the sequence of the ASO probe that will allow the optimal distinction between two alleles that differ at a single nucleotide position using ASO hybridization. These parameters must be established empirically and separately for each SNP. Consequently, there is no single set of reaction conditions that would be optimal for genotyping all SNPs, which makes the design of multiplex assays based on hybridization with ASO probes an almost impossible task.

A widely known approach to circumvent the difficult problem of assay design is to carry out multiplex ASO hybridization reactions on microarrays that carry tens or even hundreds of ASO probes for each SNP to be analysed. The probes include all possible sequences at the sites of the SNP and a stretch of nucleotide sequence that flanks the SNP<sup>39</sup>. It becomes feasible to have large numbers of ASO probes per SNP when high-density arrays are used that can carry up to  $10^6$  probes  $\text{cm}^{-2}$ , and that can be manufactured with sophisticated light-directed combinatorial synthesis<sup>40</sup>. In the GeneChip assay (Affymetrix), a computer algorithm is then used

## REVIEWS

**PEPTIDE NUCLEIC ACID (PNA).** Biopolymer molecule that consists of DNA bases connected by a backbone of peptide bonds instead of phosphodiester bonds as in natural DNA.

**LOCKED NUCLEIC ACID (LNA).** DNA analogues in which the 2' and 4' positions in a furanose ring are connected by a methylene moiety.

to interpret the complex fluorescence patterns formed by the multiple probes and to assign the genotypes of each SNP. However, mapping studies, in which 400–500 SNPs were analysed using high-density ASO microarrays, showed that the assay failed to distinguish between heterozygous and homozygous SNP genotypes for a large fraction of the SNPs despite a redundancy of 40–50 probes per SNP<sup>41,42</sup>.

One approach to carrying out ASO hybridization at conditions that allow more robust SNP genotyping is to monitor the duplexes between an ASO probe and its target over a temperature gradient, during which the optimal stringency for discriminating between the SNP alleles will be achieved at one point of the

gradient. This concept is used for parallel analysis of duplexes on microarrays with probes immobilized in miniaturized polyacrylamide gel pads<sup>43</sup> and in a dynamic allele-specific hybridization (DASH) method in a microtitre plate format<sup>44</sup>. A related approach is to use electric field strength instead of temperature to denature the ASO-probe–target hybrids<sup>45</sup>. Other approaches to increase the power of ASO hybridization are to use PEPTIDE NUCLEIC ACID (PNA) analogues<sup>46,47</sup> or LOCKED NUCLEIC ACIDS (LNA)<sup>48</sup> that have very high affinities for complementary DNA. The high affinity makes it possible to use shorter PNA and LNA probes than the natural ASO probes to improve the discrimination between the SNP alleles.

Table 2 | **Features of SNP-genotyping methods**

Method	Most significant advantage	Disadvantage	Special feature
<b>Hybridization methods</b>	Widely used	Limited genotype discrimination	
Reverse dot blot <sup>28</sup>	Multiplexing possible	Prone to non-specific background	Precursor to microarrays
GeneChip microarrays <sup>39</sup>	Very high probe density	High failure rates <sup>41,42</sup> , expensive	High-tech manufacturing process <sup>40</sup>
DASH <sup>44</sup>	Inexpensive labelling method	Complex design rules	Dedicated instrument
PNA <sup>46</sup> and LNA <sup>48</sup> probes	Specific as ASO probes	Not widely available	Innovative chemical design
TaqMan <sup>49,50</sup>	Simplicity of assay	Expensive probes	Quantitative real-time PCR
Molecular Beacons <sup>51,52</sup>	Simplicity of assay	Expensive probes	Versatile stem-loop structure
<b>Allele-specific PCR</b>	Real-time PCR assay	Requires optimization	
Intercalating dye <sup>60</sup>	Inexpensive labelling method	Non-specific products detected	Accurate quantification <sup>138</sup>
FRET primers <sup>61,62</sup>	Simplicity of assay	Expensive primers	Universal FRET primers
AlphaScreen <sup>64</sup>	Detects specific product	Expensive probes	Applicable to ASO hybridization
<b>Primer extension</b>	Accurate genotyping	Similar reagents as in PCR	
SNPstream, GBA <sup>68</sup>	Inexpensive and robust	Multiple detection steps	Automated high-throughput process
Multiplex minisequencing, SNaPshot <sup>69,71</sup>	Multiplexing capacity	Size separation step	Compatible with capillary DNA sequencers
Pyrosequencing <sup>72,74</sup>	Sequencing of up to 50 bases	Expensive, difficult to multiplex	Dedicated instrument
MassEXTEND, MassArray <sup>75</sup>	Labelling method avoided	Expensive instrument	Multiplexing capacity <sup>76</sup>
GOOD assay <sup>77</sup>	Labelling method avoided	Multi-step procedure	Sensitive mass spectrometric detection
Microarray miniseq, APEX <sup>37,70</sup>	Potential for high throughput	Requires microarray instruments	Four-colour or single-colour detection
Microarray primer extension <sup>82</sup>	Potential for high throughput	Requires microarray instruments	'Array of arrays' format
'Tag' arrays <sup>85,86</sup>	Flexible assay design	Requires microarray instruments	Generic 'tag' microarrays
Coded microspheres <sup>87</sup>	Multiplexing potential	Microspheres not widely available	Flow cytometric detection
TDI, fluorescence polarization <sup>94</sup>	Simplicity of assay	Difficult to multiplex	Universal detection principle
<b>Oligonucleotide ligation</b>	Variety of assay formats	Multiple labelled probes required	
Colorimetric OLA <sup>95,96</sup>	Robust assay	Multiple detection steps	Resembles ELISA
Sequence-coded OLA <sup>97</sup>	Multiplexing capacity	Size separation step, expensive probes	Polymer for sequence coding
Microarray ligation <sup>100</sup>	Potential for high throughput	Requires microarray instruments	Universal zip code microarray
Ligase chain reaction <sup>102</sup>	Alternative to PCR	Lower efficiency than PCR	Thermostable ligase
Padlock probes <sup>108</sup>	Localized detection	Probes difficult to produce	Avoids target amplification
Rolling circle amplification <sup>98</sup>	Signal amplification	Steric hindrance on solid phases	Based on circularized oligonucleotides
<b>Endonuclease cleavage</b>			
Restriction site analysis	Inexpensive, requires no equipment	Not suitable for high throughput	Traditional method
Invader assay <sup>112</sup>	PCR amplification avoided	Requires large amount of DNA	Interesting FLAP endonuclease

APEX, arrayed primer extension; ASO, allele-specific oligonucleotide; DASH, dynamic allele-specific hybridization; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; GBA, genetic bit analysis; LNA, locked nucleic acid; OLA, oligonucleotide ligation assay; PNA, peptide nucleic acid; TDI, template-directed incorporation.

### Homogeneous hybridization assays

At present, the most widely used ASO hybridization methods distinguish between the SNP alleles in real time during PCR in homogeneous, solution-phase hybridization reactions with fluorescence detection. The TaqMan<sup>TM</sup> (Applied Biosystems)<sup>49,50</sup> or Molecular Beacon probes<sup>51,52</sup>, which were originally designed for quantitative PCR analysis, can also be applied to SNP genotyping.

The TaqMan<sup>TM</sup> and Molecular Beacon assays are both based on a principle of energy transfer in which fluorescence is detected as a result of a change in physical distance between a reporter fluorophore and a quencher molecule on hybridization of the ASO probe to its perfectly matched target sequence (BOX 2). The strong tendency of the Molecular Beacon probes to adopt a stem-loop structure destabilizes mismatched hybrids, increasing their power of allele distinction compared with linear ASO probes<sup>51,53</sup>. Analogously to PNA and LNA probes, TaqMan<sup>TM</sup> probes modified with minor groove-binder molecules that increase their affinity for the target show improved powers of allele discrimination<sup>54</sup>. The use of two probes, each labelled with a different reporter fluorophore, allows both SNP alleles to be detected in a single tube. The TaqMan<sup>TM</sup> assay has been multiplexed by using probes labelled with seven different fluorophores<sup>55</sup>, and wavelength-shifting Molecular Beacons have been used for multiplex genotyping of up to ten SNPs<sup>56</sup>. Wavelength-shifting Molecular Beacons contain a harvester fluorophore that absorbs energy from a monochromatic light source, an emitter fluorophore with the desired emission wavelength and a quencher. In the absence of a target the probes are dark, whereas in the presence of a target the harvester fluorophore transfers energy to the emitter fluorophore that emits it as fluorescence of its own characteristic wavelength. Because the efficiency of FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) depends on the distance between the harvester and emitter fluorophore, placing several emitter fluorophores on each probe at different distances from the harvester creates possibilities for the combinatorial design of distinct fluorescence emission signatures for highly multiplexed assays<sup>57</sup>.

In the TaqMan<sup>TM</sup> and Molecular Beacon assays, the increase in fluorescence due to accumulating PCR product is usually monitored in real time in 96-well or 384-well microtitre plates. Alternatively, the fluorescence generated from the two alleles can be measured after completion of the PCR<sup>58</sup>. In this case, the results are expressed as a signal ratio that reflects the hybridization of the two oligonucleotides to the target sequence and so differences in amplification efficiency between samples do not affect interpretation of the genotyping results. Because no post-PCR processing or label-separation steps are required, the TaqMan<sup>TM</sup> and Molecular Beacon assays are simple to do, which renders them useful for high-throughput genotyping<sup>32,33</sup>. The optimal probes must be designed individually for each SNP, and the TaqMan<sup>TM</sup> and Molecular Beacon assays are therefore most efficient when a limited number of SNPs are

analysed in a large number of samples. The cost of probes modified with fluorescent and quenching moieties might also be a limiting factor in high-throughput application of the TaqMan<sup>TM</sup> and Molecular Beacon assays. An interesting advantage of the Molecular Beacon probes over the TaqMan<sup>TM</sup> probes is the possibility of immobilizing them on microarray surfaces for the detection of unlabelled DNA targets<sup>59</sup>.

### Homogeneous allele-specific PCR

PCR primers with the 3'-end complementary to either of the nucleotides of a SNP can be used in combination with a common reverse PCR primer to selectively amplify the SNP alleles<sup>31</sup> (BOX 1). The simplest approach for monitoring the formation of allele-specific PCR products using a homogeneous assay format is to include a fluorescent dye that intercalates with the double-stranded PCR products in the reaction mixture<sup>60</sup>. The Molecular Beacon probes described above have been adapted to allele-specific PCR primers for homogeneous SNP-genotyping assays<sup>61,62</sup>. The incorporation of the primer into the PCR product releases the fluorescent label from the action of the quenching molecule.

Allele-specific PCR has been rationalized by using primary allele-specific PCR primers that contain a universal 5'-tail sequence that becomes part of the PCR product on amplification. A universal pair of secondary-energy-transfer-labelled, hairpin-structured primers can therefore be used for all SNPs<sup>63</sup> (BOX 2). When using intercalating dyes or labelled allele-specific PCR primers without a consecutive target-specific detection reaction or size-separation step, the specificity of the method might be hampered owing to PRIMER DIMERS and other spurious amplification products that will not be distinguished from the actual PCR products. The homogeneous AlphaScreen (Packard Bioscience) proximity assay avoids this problem as it is based on a pair of bridging energy transfer probes that hybridize to the region between the primers in the allele-specific PCR products<sup>64</sup>. Alternatively, size separation in a high-throughput mode might be accomplished by using 96-channel capillary sequencing instruments, microplate array diagonal electrophoresis<sup>65</sup> or capillary array electrophoresis microplates<sup>66</sup>. A limitation of all variants of allele-specific PCR is that the reaction conditions or primer design for selective allele amplification must be optimized empirically for each SNP. Like the TaqMan<sup>TM</sup> and Molecular Beacon assays, the homogeneous allele-specific PCR methods are best suited for the analysis of a limited number of SNPs in large sample collections.

### DNA-polymerase-assisted genotyping

In methods based on single nucleotide primer extension — minisequencing — the distinction between genotypes of the SNPs is based on the high accuracy of nucleotide incorporation by the DNA polymerases<sup>36,67</sup> (BOX 1). The primer extension reaction is robust, allowing specific genotyping of most SNPs at similar reaction conditions. These features are advantageous for high-throughput applications because the effort required for assay design and optimization are minimized.

#### FLUORESCENCE RESONANCE ENERGY TRANSFER

A phenomenon by which the energy from an excited fluorophore is transferred to an acceptor molecule at short (<100 Å) distances, leading to quenching of the fluorescence. The efficiency of energy transfer depends strongly on the distance between the donor and acceptor molecules.

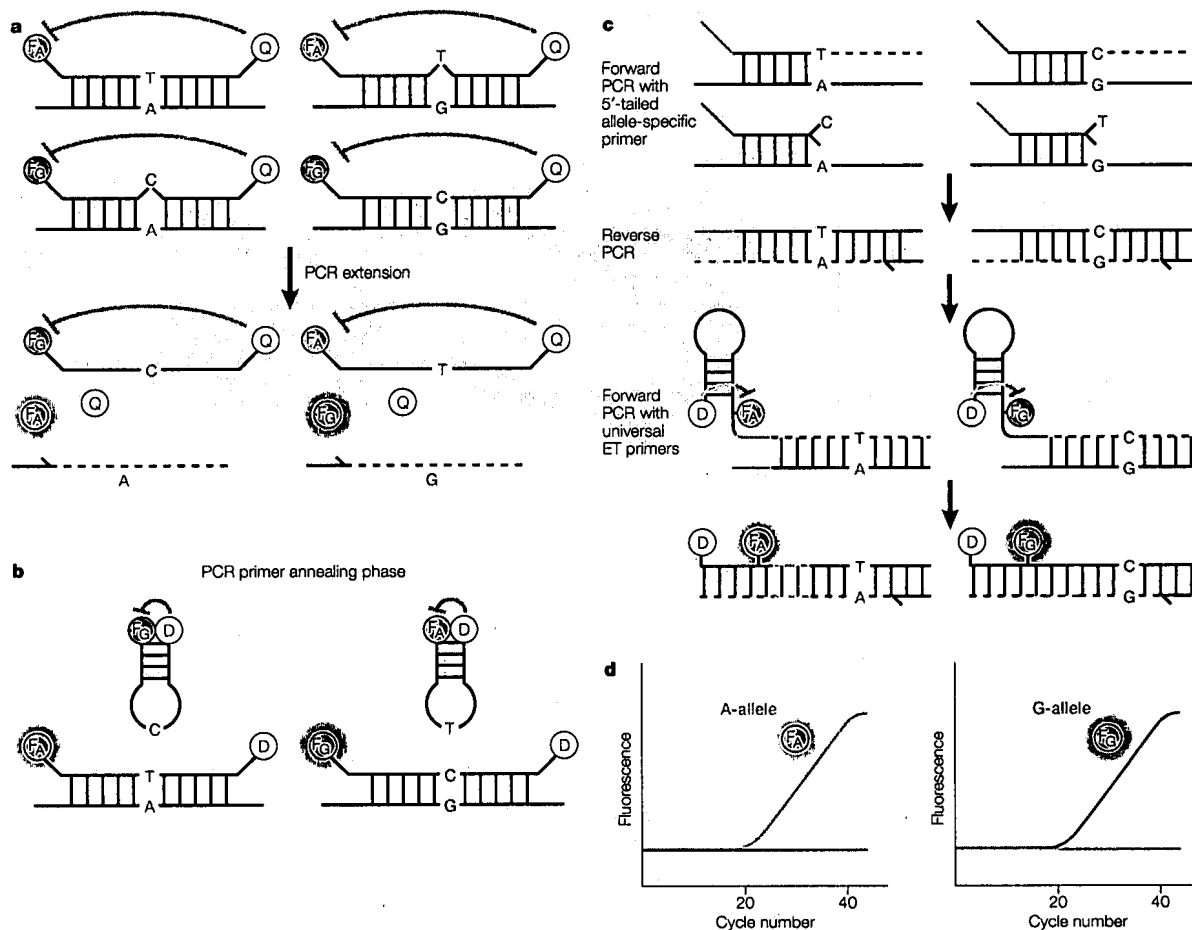
#### PRIMER DIMER

Unwanted PCR products formed when two primers interact during the extension phase of PCR, followed by extension of the 3'-end of one or both primers with the other primer acting as a template.



## Box 2 | Principles for homogeneous SNP genotyping by real-time PCR

The example illustrates the detection of an A-to-G transition. Panel a shows the 5'-exonuclease (TaqMan™) assay: allele-specific oligonucleotide probes are labelled with different fluorophores ( $F_A$  (blue) or  $F_G$  (red)) at their 3'-ends and with a quencher molecule (Q) at their 5'-ends. The quencher interacts with the fluorophores by fluorescence resonance energy transfer, quenching their fluorescence. The allele-specific probes are included in the PCR reaction mixture. During the annealing phase of PCR, the probes hybridize to the strands of the PCR products and during the extension phase of PCR, the 5'-3' exonuclease activity of the DNA polymerase degrades perfectly matched, annealed probes. The fragmented probes are released into the solution, separating the fluorophore from the quencher, which leads to an increase in fluorescence. Mismatched probes are displaced from the target without degradation. Molecular Beacon probes (shown in panel b) consist of a sequence that is complementary to the target sequence and a short stretch of self-complementary 5'- and 3'-nucleotides with a fluorophore ( $F_A$  or  $F_G$ ) at the 5'-end and a DABCYL moiety as quencher (D) at the 3'-end. When free in solution, the Molecular Beacon probes adopt a stem-loop structure that brings the fluorophore and quencher into close proximity. When a Molecular Beacon probe hybridizes to a perfectly matched target during the primer annealing phase of PCR, the stem-loop structure opens, and the distance between quencher and fluorescent molecule increases, which restores the fluorescence. Mismatched probes readily adopt the stem-loop structure. It should be noted that the physical energy transfer mechanism differs between the TaqMan™ probes, with the fluorophore TAMRA as quencher, and the Molecular Beacon probes, with DABCYL as quencher. Universal allele-specific energy transfer primers are shown in panel c. The primary allele-specific PCR primers carry different 5'-tail sequences. The secondary primers consist of a 3'-sequence complementary to the tail sequences and a sequence that is held in a hairpin loop conformation by complementary stem sequences similar to the Molecular Beacon probes. During the primary allele-specific PCR, the 5'-tail sequence of the primer becomes incorporated into the PCR product. The energy transfer primer is used to initiate a secondary PCR. On synthesis of the reverse strand of the PCR product, the stem-loop structure of the secondary primers opens, separating the quencher from the fluorophore, and therefore restoring its fluorescence. Fluorescence measurement is shown in panel d. The increase in fluorescence at the emission wavelengths for the fluorophores  $F_A$  and  $F_G$  is monitored in real time during PCR. At a threshold cycle, the fluorescence rises to a detectable level and increases as more PCR products accumulate. Alternatively, the result can be interpreted by measurement of fluorescence intensity at the end point of PCR. (DABCYL, 4-dimethylaminoazobenzene-4'-sulphonyl; ET, energy transfer; TAMRA, 6-carboxytetra-methylrhodamine.)



Consequently, single nucleotide primer extension is gaining acceptance as the reaction principle of choice for high-throughput genotyping of SNPs, and has been adapted to various assay formats, detection strategies and technology platforms (FIG. 1).

In an ELISA-like single nucleotide primer extension assay, incorporated HAPTEN-labelled nucleotide analogues are detected colorimetrically (genetic bit analysis, Orchid Biosciences)<sup>68</sup>. In this indirect detection procedure, the incorporated haptens are recognized by antibodies, and enzymes that are conjugated to the antibodies catalyse the formation of a coloured product. This detection strategy in a 384-well microtitre plate format has been automated for production-scale SNP genotyping (SNPstream, Orchid Biosciences). DNA-sequencing instruments might also be used as platforms for genotyping SNPs using fluorescently labelled dideoxynucleotides in the minisequencing reactions. The electrophoretic size-separation step facilitates multiplex genotyping in the range of tens of SNPs per reaction using primers modified with 5'-tails of varying length<sup>69–71</sup> (SNaPshot, Applied Biosystems), and by using 96-channel capillary sequencers, this assay can be automated for high-throughput genotyping.

In the PYROSEQUENCING (Pyrosequencing AB) method, primer extension is monitored by enzyme-mediated luminometric detection of pyrophosphate, which is released on incorporation of deoxynucleotide triphosphates<sup>72,73</sup>. The genotype of a SNP is deduced by sequential addition and degradation of the nucleotides using apyrase in a dedicated instrument that operates in a 96-well or 384-well microtitre plate format<sup>74</sup>. Using pyrosequencing, short 30–50-bp sequences of DNA that flank a SNP can be determined. A limitation of the method is that the sequential identification of bases prevents genotyping of several SNPs per reaction in diploid genomes.

Mass spectrometry (MALDI-TOF, matrix-associated laser desorption time-of-flight mass spectrometry) is used to detect the primer extension products in two closely related SNP-genotyping assays — the PROBE (primer oligo base extension) assay<sup>75</sup> (now called MassEXTEND, Sequenom) and the PinPoint assay<sup>77</sup> (Applied Biosystems). Mass spectrometry is particularly useful as a read-out method for primer extension reactions because primers of different lengths can be used in combination with mixtures of deoxy- and dideoxynucleoside triphosphates designed to yield allele-specific primer extension products with clear differences in their molecular mass. Moreover, the assays can be multiplexed if the primer extension products for each SNP have non-overlapping mass distributions<sup>76</sup>. A difficulty with MALDI-TOF is that the primer extension products must be rigorously purified before measurement to avoid background from biological material present in the sample. This limitation is avoided in the so-called 'GOOD' assay<sup>77</sup> by increasing the sensitivity of the mass spectrometric detection so that a small aliquot of the extended primer is sufficient for measurement. The increase in detection sensitivity is accomplished by introducing

thiol groups into the 3'-region of the primer, which allows the mass of the allele-specific products to be reduced by enzymatic digestion of the 5'-end of the primer, and by neutralizing the negative charge of DNA by alkylating the thiol groups<sup>77,78</sup>.

Owing to its high sequence specificity and robustness, the DNA-polymerase-assisted single nucleotide primer extension reaction is well suited to highly parallel genotyping of SNPs on microarrays. In a comparison with ASO hybridization reactions in the same microarray format, the minisequencing reaction provided tenfold better power of discrimination between genotypes than hybridization with ASO probes<sup>37</sup>. For minisequencing on microarrays<sup>37</sup>, also denoted 'arrayed primer extension' (APEX)<sup>70</sup>, one detection primer for each SNP is immobilized covalently on a microscope slide. After an extension reaction using fluorescent dideoxynucleotides, the array is analysed by fluorescence scanning<sup>79–81</sup> (BOX 3). Instead of an extension reaction using one primer per SNP and four fluorescent dideoxynucleotides, two immobilized allele-specific primers per SNP can be extended using a mixture of natural and fluorescent deoxynucleotides with RNA<sup>82</sup> or DNA<sup>83,84</sup> as the template for the reaction (BOX 3). An array of arrays format, in which separate reaction chambers are formed on an array by a silicon rubber grid, allows simultaneous genotyping of up to 300 SNPs in 80 samples by minisequencing or allele-specific primer extension<sup>82</sup> (FIG. 2). This assay can yield more than 10,000–20,000 genotypes per microscope slide and the automation of this format would allow extremely high-throughput genotyping. A flexible strategy for SNP genotyping using microarrays is to carry out cyclic primer extension reactions in solution with specific primers that are tailed with 5'-tag sequences, and to use microarrays with complementary oligonucleotide tags for hybridization-based capture and sorting of the products of the cyclic minisequencing reactions<sup>85,86</sup> (BOX 3). This strategy has been used in conjunction with low<sup>85</sup> and high<sup>86</sup> (GeneFlex, Affymetrix)-density microarrays. These microarray-based methods are particularly suited to the analysis of large panels of SNPs.

The concept of generic tags for capturing products of cyclic primer extension reactions has been applied to arrays of MICROSPHERES. Each class of microsphere is embedded with fluorophores that have a characteristic emission wavelength, and the microspheres in each class carry a unique complementary 'tag' sequence for capturing the SNP-specific primer extension products. Measurement of the fluorescence of each individual microsphere in a flow cytometer allows determination of which class it belongs to, and the genotype of the SNPs is determined by the captured fluorescent product<sup>87,88</sup>. So far, this assay has been multiplexed for the detection of tens of SNPs. Microspheres with up to 100 distinct spectral characteristics, generated by mixing fluorophores with red and infrared emission, are available<sup>89</sup> (Luminex).

The accuracy of the primer extension reaction also allows quantitative determination of allelic ratios of SNPs in pooled DNA samples<sup>90</sup>. Particularly accurate quantification is achieved using <sup>3</sup>H-labelled

#### ELISA

(enzyme-linked immunosorbent assay). A widely used immunochemical method for detecting antigens or antibodies. ELISA methods are carried out in microtitre plates and use colorimetric detection.

#### HAPTEN

Small molecule that is able to invoke an antibody response when used for immunization of an animal.

#### PYROSEQUENCING

A method for DNA sequencing, in which the inorganic pyrophosphate (PPi) that is released from a nucleoside triphosphate on DNA chain elongation is detected by a bioluminometric assay.

#### MICROSPHERES

(also known as microparticles or microbeads). Small 1–100-µm diameter particles used as solid supports in bioassays. They can carry a probe or primer, and can contain internal magnetic compounds to allow magnetic separation or internal fluorescent compounds for labelling.

# FLUORESCENCE POLARIZATION

A detection method based on excitation of a fluorescent molecule by plane-polarized light, and measurement of the rate of depolarization of fluorescence. This rate is proportional to the rate of tumbling of a fluorescent molecule. As small molecules tumble faster than large molecules in solution, fluorescent molecules of different sizes can be distinguished.

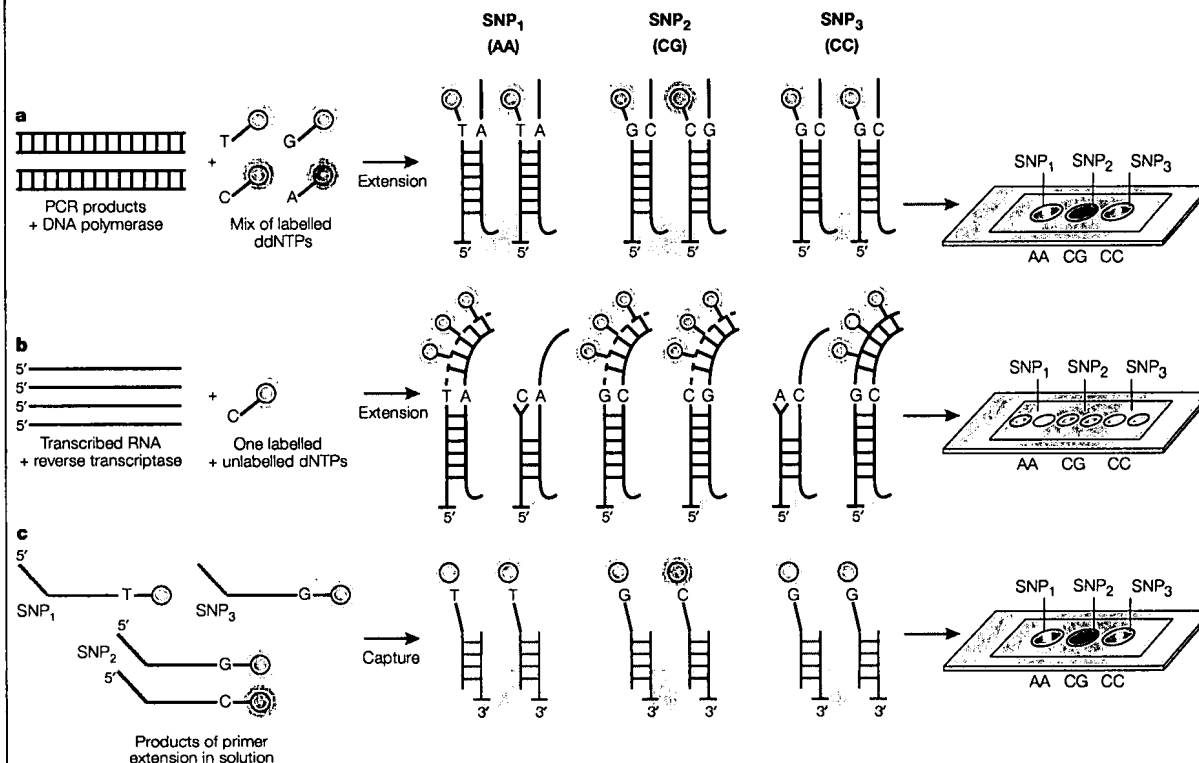
deoxynucleotides that are chemically similar to the natural nucleotides, and are, therefore, incorporated with high specificity by the DNA polymerase<sup>90,91</sup> or using mixtures of unlabelled deoxy- and dideoxynucleotides followed by mass-spectrometric detection<sup>92</sup>. Analysis of pooled DNA samples is beneficial for increasing throughput in association studies and for determining the allele frequencies of SNPs in various populations.

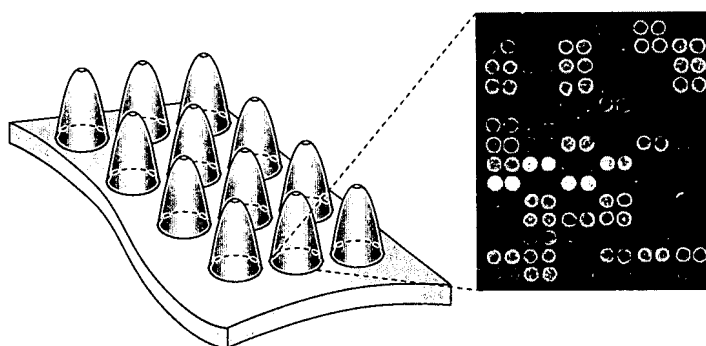
Because both the PCR and primer extension reaction mixtures contain primers, nucleoside triphosphates and a DNA polymerase, a step to remove or inactivate excessive PCR reagents is crucial for the success of all primer extension methods. Immobilizing

either the SNP-containing templates or the detection primers on a solid support, such as microtitre plate wells<sup>68,90</sup>, microparticles<sup>74,88</sup> or microarrays<sup>37</sup>, followed by washing the solid support, provides an efficient way to remove excessive reagents and to render the template single stranded before the primer extension reaction. Alternatively, the PCR primers and nucleotides can be degraded enzymatically with alkaline phosphatase and an exonuclease before the genotyping reaction<sup>93</sup>. The enzymatic degradation step has allowed the design of a technically simple and robust homogeneous SNP-genotyping assay based on cyclic primer extension reactions and detection by FLUORESCENCE POLARIZATION<sup>94</sup>.

## Box 3 | Strategies for SNP genotyping by primer extension using microarrays

The genotyping of three single nucleotide polymorphisms (SNPs) is illustrated (SNP<sub>1</sub> with the nucleotide variation A/G; SNP<sub>2</sub> with G/C; and SNP<sub>3</sub> with C/T) for a sample with the genotypes AA, CG and CC. Panel a in the figure shows minisequencing — arrayed primer extension. One primer for each SNP to be genotyped is immobilized covalently on the surface of a microscope slide. Multiplex PCR products that span the SNP sites, a mixture of fluorescently labelled terminating nucleotide analogues (ddNTPs, dideoxynucleoside triphosphates) and a DNA polymerase are added to the arrays. The primer extension reactions are allowed to proceed on the array surface, and the microscope slides are scanned. The positions of the primers on the microarray surface define which SNP is analysed and the fluorescent nucleotide(s) by which a primer becomes extended defines the genotype of the SNP. Allele-specific primer extension is shown in panel b. Two allele-specific primers with the 3'-base complementary to the two possible nucleotides of each SNP are immobilized on the array. The multiplex PCR products that span the SNPs are transcribed into numerous RNA copies by an RNA polymerase. The RNA molecules act as templates for a primer extension reaction catalysed by a reverse transcriptase, in which several fluorescent deoxynucleotides (dNTPs, deoxynucleoside triphosphates) become incorporated in each product. For homozygous genotypes, a signal is generated from one of the allele-specific primers, and for heterozygous genotypes a signal is generated from both primers. Primer extension using tag arrays is shown in panel c. Cyclic single nucleotide primer extension reactions are carried out in solution in the presence of fluorescently labelled dideoxynucleotides using primers carrying an extra tag sequence in their 5'-end. Generic arrays of oligonucleotides that are complementary to the 'tags' of the primers are used to capture the products of the cyclic minisequencing reactions.





**Figure 2 | SNP genotyping by minisequencing using an 'array of arrays'.** Each small array comprises a set of primers for detecting single nucleotide polymorphisms (SNPs), and each array is isolated by a silicon rubber cone that forms an individual reaction chamber for the array<sup>82</sup>. Reagents are introduced through a hole in the tip of the cone. A standard microscope slide can hold up to 80 arrays. A fluorescence image of one array is shown, in which minisequencing reactions for 84 SNPs with the primers spotted in duplicate have been carried out with TAMRA-labelled ddGTP (U. Liljedhal and A.-C. Syvänen, unpublished data). ddGTP, dideoxyguanosine triphosphate; TAMRA, 6-carboxytetramethylrhodamine.

### Ligation methods

The discrimination by DNA ligases against mismatches at the ligation site in two adjacently hybridized oligonucleotides is the basis for genotyping of SNPs by the oligonucleotide ligation assay (OLA)<sup>39</sup> (BOX 1). This assay has been combined with colorimetric detection in microtitre plate wells<sup>95,96</sup> and with multiplex detection using fluorescently labelled ligation probes with different electrophoretic mobilities that can be analysed in a DNA-sequencing instrument<sup>97</sup>. OLA has also been used in microarray formats with one of the ligation probes immobilized<sup>98</sup> or with immobilized single stem-loop probes<sup>99</sup>. Alternatively, ligation can be carried out in solution followed by capture of the ligation products on microarrays<sup>100</sup> or on microparticles<sup>101</sup> that carry a generic set of oligonucleotides that are complementary to a 'tag' sequence on one of the ligation probes.

In the ligase chain reaction, two pairs of oligonucleotide probes are used in cyclic ligation reactions, together with a thermostable DNA ligase for exponential amplification of genomic DNA<sup>102</sup>. In practice, the thermostable ligases<sup>103</sup> are more frequently used for genotyping SNPs in combination with PCR before the allele-specific ligase detection reactions<sup>104,105</sup>. Because the reaction mechanisms for PCR and ligation are different, the reagents for both reactions can be combined. This feature is used in a homogeneous, real-time PCR assay with ligase-mediated genotyping and detection by FRET<sup>106</sup>. Compared with DNA-polymerase-assisted primer extension methods, a drawback of the OLAs is that detection of each SNP requires three oligonucleotides (BOX 1), of which one is 5'-phosphorylated, and two carry detectable labels, which obviously increases the costs of these assays.

Padlock probes are linear oligonucleotides, the ends of which are complementary to the target and have a central stretch of random sequence<sup>107</sup>. When perfectly hybridized to their target sequence, padlock probes can

be circularized by ligation, whereas a mismatch with the target sequence prevents ligation. Padlock probes have been used for colorimetric *in situ* detection of single-base variations in repetitive  $\alpha$ -satellite DNA in human metaphase chromosomes<sup>108</sup>. Circularized oligonucleotides can act as templates for DNA-polymerase-assisted rolling circle amplification (RCA)<sup>98,109</sup>. RCA can therefore be used to amplify the number of ligated circularized padlock probes to a level required for detecting single-copy sequences<sup>110</sup>. A homogeneous, isothermal assay for genotyping individual SNPs in a microtitre plate format has been devised by combining exponential amplification of ligated padlock probes using a branched rolling circle amplification reaction with detection by energy-transfer-labelled hairpin primers based on Molecular Beacons<sup>111</sup>.

### Invasive cleavage of oligonucleotide probes

The Invader assay (Third Wave Technologies) makes use of two target-specific hybridization oligonucleotides — an allele-specific signalling probe with a 5'-region that is non-complementary to the target sequence and an upstream invader oligonucleotide<sup>112</sup> (BOX 1). When the allele-specific probe is perfectly matched at the SNP, the three-dimensional structure formed by these two oligonucleotides and the target sequence at the site of the SNP is recognized and is cleaved by a 5'-endonuclease, called FLAP endonuclease, that is specific for this particular DNA structure. This cleavage releases the 5'-sequence of the signalling probe, which can be detected directly<sup>112</sup> or further amplified by a serial, isothermal Invader assay based on a FRET-labelled probe<sup>113</sup>. The Invader assay has been combined with mass spectrometric detection<sup>46</sup> and with detection by fluorescence polarization<sup>114</sup>. In principle, the serial Invader assay can be applied for identifying SNPs in genomic DNA without previous PCR amplification, but a limitation of the assay is that it requires a large amount of target DNA. Therefore, the homogeneous Invader assay has been applied for genotyping SNPs in DNA fragments previously amplified by PCR<sup>115</sup>. The assay has been adapted to a solid-phase format, which is a prerequisite for multiplex genotyping using the Invader principle in a microarray format<sup>116</sup>.

### Future trends

In practice, the requirement of a PCR amplification step to achieve sensitive and specific SNP genotyping is the principal factor that limits the throughput of assays today. Multiplex PCR amplification of more than ten DNA fragments is difficult to carry out reproducibly owing to the generation of spurious amplification products<sup>39,41,42,80</sup>. Recently, a PCR strategy based on a single universal PCR primer for amplification of a reduced representation of the human genome that avoids the problem of carrying out multiplex PCR was applied to SNP discovery and validation<sup>117</sup>. Similar generic amplification strategies could also be devised for genotyping panels of known SNPs to circumvent multiplex PCRs with locus-specific primers. Another suggested approach for avoiding non-specific primer interactions during multiplex PCR is to carry out

the amplifications with primers immobilized at physically distinct locations in 'colonies' (PCR colonies)<sup>118</sup>, gel pads<sup>119</sup> or using microelectronic arrays<sup>120</sup>. SNP genotyping by mini-sequencing<sup>67</sup> or pyrosequencing<sup>73</sup> would be compatible with these formats.

The ligation and invasive cleavage methods described above<sup>109–113</sup> make use of two recognition events between oligonucleotides and their targets and so, in principle, these methods have the required specificity for allele-specific SNP detection in unamplified genomic DNA. To achieve the desired sensitivity, the methods rely on enzymatic amplification of the signals. As the ligation and invader methods are based on several enzymes and fluorescent detector probes labelled with multiple fluorophores, it remains to be seen whether they can contest the standard PCR-based methods in terms of reagent cost, throughput and accuracy.

New PCR instruments that use microcapillaries instead of microtitre plate formats have been devised, and offer increased PCR throughput and reduced reagent costs as they use extremely short amplification times and small reaction volumes<sup>121,122</sup>. Fully automated SNP analysis systems could then be designed based on homogeneous detection, or by streamlining the PCR and the subsequent genotyping procedure in microfluidic 'lab chip' devices that operate with submicrolitre reaction volumes. Such microfluidic devices are now under development in several biotech companies<sup>123</sup>.

Recent developments of composite materials and fluorescence detection strategies offer increased detection sensitivity and specificity for SNP-genotyping assays. Hybrid gold and silver nanoparticles have been used, instead of fluorophores, as labels on allele-specific oligonucleotide probes<sup>124</sup>. Gold nanoparticles can also replace the organic DABCYL moieties as quenchers on Molecular Beacon energy transfer probes<sup>125</sup>, enhancing the sensitivity of the assays by two orders of magnitude. A recently described microvolume detection technique based on TWO-PHOTON EXCITATION can potentially be used to detect individual microparticles in multiplexed bioassays<sup>126</sup>. Another approach to highly multiplexed

assays is to use fibre-optic sensors to detect large numbers of coded microspheres in real time<sup>127</sup>. For SNP genotyping, coded microspheres that carry 'tag' sequences would be captured in miniaturized wells at the ends of fibre-optic detectors, and the signals that originate from hybridization, primer extension or oligonucleotide ligation reactions could be monitored (Illumina). Finally, in another very promising strategy for multiplexing bioassays, multicolour optical coding is accomplished by embedding different sized QUANTUM DOTS into polymeric microbeads at precisely controlled ratios<sup>128</sup>. Because of the unique spectral properties of the quantum dots, this technology has the potential for several-thousand-fold multiplexing.

Despite the numerous technical advances in detection and multiplexing strategies, no technique clearly represents a breakthrough. It is difficult to predict which, if any, existing SNP-genotyping technology will facilitate the required 100- or 1,000-fold increase in throughput required for whole-genome SNP analysis in sample collections of relevant size. The next generation technology for SNP analysis should avoid the PCR amplification step, and the technology could be based on the analysis of single DNA molecules, which would allow direct determination of the haplotypes formed by the SNPs. Such futuristic candidate technologies might derive from a further development of the system for haplotyping by carbon nanotube atomic force microscopy probes<sup>129</sup> or a further refinement of the microscopic technology that has recently allowed direct optical mapping of the whole *Escherichia coli* genome<sup>130</sup>. Hopefully, future SNP-genotyping technology will be more elegant than the incremental and brute-force expansion of existing technologies in the way that the Sanger sequencing method has been exploited for genome sequencing. One thing is certain — once very-high-throughput SNP genotyping is available, this technology will have a profound impact on our understanding of the relationship between genetic variation and biological function.

**TWO-PHOTON EXCITATION**  
A detection system in which excitation of fluorophores takes place only in a small three-dimensional focal volume.

**QUANTUM DOT**  
Nanocrystal that consists of a core of cadmium selenide wrapped with multiple monolayers of zinc sulphide that have several times higher extinction coefficients than organic fluorophores. The quantum dots can be excited with light of a single wavelength, and emit very bright fluorescence at several wavelengths that are determined by the size of the cadmium selenide core.

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